BIOCONVERSION OF MSW PAPER TO FUEL ETHANOL: A WASTE REDUCTION REPORT

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A WASTE REDUCTION REPORT

Report prepared by:

Morris Wayman
Department of Chemical Engineering and Applied Chemistry
University of Toronto
Toronto, Ontario

ACKNOWLEDGEMENT AND DISCLAIMER

This report was prepared for the Ontario Ministry of Environment and Energy (formerly Ministry of the Environment) as part of a Ministry funded project. The views and ideas expressed in this report are those of the author and do not necessarily reflect the views and policies of the Ministry of Environment and Energy, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The Ministry, however, encourages the distribution of information and strongly supports technology transfer and diffusion. Note, all references to Ministry of the Environment in this report should read Ministry of Environment and Energy.

June 30, 1992

Mr. Dan Ionescu
Senior Project Coordinator
Waste Reduction Office
Ontario Ministry of the Environment
101 Bloor Street West - 14th floor
Toronto, Ontario
M5S 2Z7

Dear Mr. Ionescu,

PROJECT 502G
BIOCONVERSION OF MSW-PAPER TO FUEL ETHANOL:
A WASTE REDUCTION PROJECT

We have pleasure in submitting herewith 3 copies of our **FINAL REPORT** under this project. Outlined in this Report are the highlights of our accomplishments under this grant, details of which are given in the Appendices. We estimate that large scale implementation of our waste paper-to-ethanol process would reduce the volume of MSW going to landfill by 40 - 50 %. Some RDF producers claim 80 - 90 %.

We have succeeded in specifying a best process for bioconversion of waste paper to ethanol, yielding 350 - 400 liters ethanol per ton of waste paper, and have outlined a better best process incorporating later knowledge which raises yields to 460 liters ethanol per ton, about 90 % of that theoretically possible.

We have provided here Flow Charts and Mass Balances at 2 levels, for a demonstration plant receiving 4 tons waste paper per day and a full scale plant processing 400 tons waste paper per day. We have calculated the profitability of the full scale plant, detailing capital costs, operating costs and income, as well as other important elements which define investment, that is working capital, engineering and management during construction and financing costs. With waste paper at zero cost, profitability is

unattractive; however, when modest tipping fees are charged, profitability becomes acceptable. An important effect on profitability would be a more realistic price for this environmentally friendly, high octane, non-petroleum motor fuel ethanol.

One of the key components in our process is the on-site manufacture of the needed cellulase enzymes. We have 2 principal accomplishments over previous workers in this field, the identification of an excellent cellulase inducer derived from waste newspaper, and the finding that inducers made from whole wheat flour supply nutrients and buffers valuable in saccharification and fermentation. Enzyme research continues.

The bioconversion process is ready for much larger scale implementation, and there are live prospects that this will take place shortly in both Western and Eastern Europe, thereby demonstrating that this is an exportable technology. Export potential should be pursued.

We have listed our main *Conclusions*, and have made *Recommendations* indicating actions to be taken to establish a new, substantial biofuels industry in Ontario.

I am grateful to the Ministry of the Environment for the opportunity to do this research. I also thank my collaborators Shu Chen and Kim Doan for their many contributions, as is apparent from their co-authorship of published papers in the Appendices. Professor Esteban Chornet, of the Universite de Sherbrooke, Sherbrooke, Quebec, deserves special thanks for his collaboration in pretreatment research. May I express the hope that the Ministry, together with other Ministries concerned such as Energy, Agriculture and Food, and Transportation will ensure that this ongoing research will receive the necessary support to enable us to solve some of the remaining problems. This Ministry has up to now led the field in its support of this important research: may it continue to do so!

Yours sincerely,

Morris Wayman

Morris Wayman
Professor

ABSTRACT

During two years of research mostly funded by the Ontario Ministry of the Environment, we have analysed and processed to ethanol representative waste papers mechanically separated from Municipal Solid Waste (MSW), waste office papers, waste paperboard packaging and other waste papers. Landfill volume reduction to be realized by bioconversion of MSW-paper to ethanol has been estimated at 40 - 50 %. Our process, enzymatic liquefaction followed by saccharification combined with fermentation produced 350 - 400 liters ethanol per ton of waste paper, or, with pretreatment, 460 liters per ton. We have also studied synthesis of the key cellulase enzyme. Mass Balances and Flow Charts for small and large industrial facilities as well as Economic Analyses and calculations of Profitability are included in this Report. We have drawn these Conclusions:

1. The segregation and collection of all waste paper in Ontario would reduce the volume of MSW going to landfill by 40 - 50 %. If a commercial MSW fractionation system such as is common in Europe and the US were in place, the reduction in volume would be 80 - 90 %. The waste paper so diverted would be used to make valuable marketable products such as ethanol.

2. There is enough MSW-paper in Ontario to displace 10 % of gasoline upon bioconversion to the environmentally friendly, high octane, non-petroleum motor fuel ethanol. Improvements in air quality, lessening of crop damage from ground level ozone and better human health can be expected.

3. Potential markets for ethanol in Ontario are large, annual motor fuel consumption being around 12,000 million liters. Substitution of 10 % of this by ethanol would provide a market of 1,200 million liters per year. To supply this market would require a new industry of 20 - 24 full size distilleries employing about 2,500 workers permanently, on site, and an equal number occupied in the supply of waste paper, and about 20,000 man years of employment in construction. Investment of about $60 million in each plant would amount to $1.2 -1.5 billion.

4. A best process for bioconversion of segregated waste papers, and of the light fraction of mechanically separated MSW, usually designated Refuse Derived Fuel (RDF) because of its high calorific content, is detailed in this Report. The process can be epitomized as enzymatic liquefaction followed by saccharification combined with fermentation by common bakers' yeast. Enzymatic saccharification is preferred to acid hydrolysis of waste paper because the conditions are milder and yields of ethanol are higher. The necessary enzymes can and should be made on site. By this best process, yields of ethanol of 350 - 400 liters/ton of waste paper, or 200 liters/ton of RDF were obtained. These are 70 - 80 % of the ethanol potential based on fermentation by bakers' yeast.

5. The yields just quoted were obtained without any pretreatment ahead of liquefaction. A very brief steaming at 200°C with 0.5 % sulphuric acid catalyst raised yields of ethanol from waste paper to 460 liters/ton, 90 % of the theoretical potential based on cellulose content and the use of bakers' yeast. Pretreatment with acid catalyst as just described has the advantages of sterilizing the waste paper, thereby avoiding bacterial contamination, and shortens fermentation times, as well as these higher yields.

6. The technology described in this Report is sufficiently advanced to form the basis for a demonstration waste paper to ethanol plant and, under favorable circumstances such as exist in Eastern Europe, a full scale plant. The technology is exportable, and plans are in progress to apply it to such enterprises in Europe, both East and West.

7. Flow Charts and Mass Balances are presented here for 2 sizes of ethanol plants, a demonstration plant accepting 4 tons/day, 1,000 tons/year of waste paper, and a full scale plant receiving 400 tons/day, 128,000 tons/year of waste paper, showing all inputs, process conditions and products.

8. The Mass Balance of the full scale plant was used for economic analysis, which gave total investment of $58.5 million including fixed capital, working capital, engineering and management during construction and financing costs. Operating costs, including maintenance and depreciation, but accepting waste paper at zero

cost, came to $8.25 million/year. Revenue, based on sale of 40 million liters of fuel ethanol at 25 cents/liter plus sale of Distillers Dried Grains Solids, amounts to $12 million/year. This gives a crude Return on Investment of only 6.4 %, not enough to attract private investors. To raise ROI to an acceptable level, 20, 25 and 30 %, requires tipping fees of $62.00, $85.60 and $108.60/ton of waste paper. An ethanol price of 30 cents/liter, which is more than justified by provincial and federal subsidies for ethanol in gasoline would reduce these tipping fees to $43, $65 and $87/ton. A smaller plant would require larger tipping fees for equivalent profitability. For example, a plant of 20 million liters ethanol annual capacity would require a tipping fee of about $60 - $65/ton for 20 % ROI. These are significantly lower tipping fees than present municipal tipping fees, and may well attract generators of waste paper such as banks to become sponsors or partners in enterprises for the bioconversion of their waste paper to ethanol.

9. Capital and operating costs for a waste paper to ethanol plant may be reduced significantly by partnering with a nearby enterprise which has available excess low pressure steam and/or electricity, such as a pulp mill or nuclear plant. Also a partner which can make more profitable use of the residues from this plant may help the economics and simplify the design.

10. Economic success depends on the use of enzymes made on site rather than purchased enzymes. In the corn syrup-glucose industry, which has many parallels, enzyme prices are low enough that most of the industry uses purchased enzymes rather than making their own. This Report includes a detailed description of the synthesis of the main cellulase enzyme using waste newspaper or other inexpensive carbon sources. In our Flow Chart for ethanol from waste paper we show 10 % of the pretreated waste paper being diverted to enzyme production. Laboratory results indicate that this figure may be much too high, and that perhaps as little as 5 % of pretreated waste paper would be needed for enzyme production.

11. A better best process, described here, would include acid catalysed pretreatment, xylose fermentation and cellobiase supply by the use of other yeasts, named in this Report. By conducting fermentation in a

cascaded series of fermenters, the application of these exotic yeasts, would be relatively simple. Xylose fermentation would raise yields by about 10 % higher than obtainable by the use of bakers' yeast, and the use of cellobiase containing yeasts would lower the cost of enzyme.

12. Motor fuel containing 5 - 10 % ethanol, marketed in southwestern Ontario by UCO Petroleums, and a small volume in Mohawk's northwestern gas stations contain only corn- or grain-based ethanol, and the ethanol plant being planned by Canadian Agra will be corn-based. In addition, there are a few small farm-based distilleries, the products being used in mobile equipment. Specific steps to encourage the building of a biofuels-from- waste paper industry are given in the Recommendations.

From these findings, we recommend:

1. The bioconversion of Ontario's waste paper to motor fuel ethanol requires the establishment of a new 20 - 25 plant industry, with investment of $1.2 - 1.5 billion. The promise of such enormous industrial activity, with accompanying employment and environmental benefits, demands serious attention. Ministries of the Environment, Energy, Agriculture and Food, and Transportation should join to encourage the development of this new industry.

2. Research and development in bioconversion of waste paper to fuel ethanol needs to be substantially increased to a level commensurate with its environmental and industrial importance. Existing facilities are inadequate. A proper pilot plant capable of solving problems of feedstock, process, microbiology and engineering is required. For example, the search for special strains or mutants of fungi, yeasts and bacteria which have beneficial characteristics should be a continuing endeavour. Consideration might be given to building on Iogen's pilot plant, or St. Lawrence Reactor's pilot plant, neither of which is adequately equipped now. Adaptation and expansion may take less time than a grass roots facility, however that is far from certain, and needs a proper assessment. The new pilot plant should be equipped to test a range of modern technologies for bioconversion of waste paper to fuel ethanol: simultaneous saccharification and fermentation, enzyme production and use, extractive fermentation,

cracked corn dehydration of 80 % (or less) ethanol concentration, cascade fermentation, xylose-fermentig yeasts, cellobiose- fermenting yeasts, immobilized yeasts for very rapid fermentation, and stillage recycling and disposal.

3. The exportability of the technology described in this Report is established by the European response. A continuing effort to expand the adoption of this technology in the US, Europe and Japan is justified and needs to be pursued.

4. Waste paper needs to be considered as much a resource as, say, spent sulphite liquor, and should be collected by waste paper generators and converted to ethanol. A consortium of banks should be encouraged to follow this route with their own waste paper. Large financial and industrial conglomerates should be encouaged to follow this pattern. Similarly, fibres in effluents from pulp mills should be treated as a source of ethanol for energy in their own operations.

5. Consideration should be given to dedicated biofuel plants based on specific segregated feedstocks such as waste paperboard packaging. Such papers have characterisitics which may benefit from modified process procedures and may do better in terms of yield and costs in such dedicated plants than when included in mixed waste paper feedstock.

6. Since the segregation and collection of waste paper is estimated to reduce the volume of MSW to landfill by 40 - 50 %, while an MSW fractionation system such as is common in Europe and the US promises to reduce that volume by 80 - 90 %, engineering studies of the costs involved in the installation of such MSW fractionation systems need to be carried out. Such a Materials Recovery Facility would produce more marketable products, and lends itself to integration with other resource recovery systems.

7. Research on improved processes for production of cellulase enzymes is a matter of some urgency, to be encouraged by the Ministry.

8. The identification or development of a yeast strain which is a good fermenter at 40°C or above should have a high priority in the research program. Fermentation by such a strain should be accompanied by vacuum distillation of the ethanol as produced.

9. Fermentation of xylose requires oxygen (or air): too little results in poor xylose fermentation, while too much results in growth of yeast at the expense of ethanol production. To optimize xylose fermentation, we need to know the proper oxygen to xylose ratio. This is a specific piece of information which should be the subject of an important item in the research program.

10. The pretreatment reported here was catalysed by sulphuric acid. The use of gaseous SO_2 or CO_2 as the acd catalyst would have many advantages, and should be studied.

11. The strategy of how feedstock is added, and perhaps of how enzyme is added, to the saccharifying medium needs to be studied for higher ethanol concentrations, thereby reducing distillation costs.

BIOCONVERSION OF MSW PAPER TO FUEL ETHANOL:
A WASTE REDUCTION PROJECT

FINAL REPORT

JULY 1, 1990 to JUNE 30, 1992

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PROJECT 502G

BIOCONVERSION OF MSW PAPER TO FUEL ETHANOL: A WASTE REDUCTION PROJECT

1. General and Specific Objectives

This Project has as its General Objective the reduction and diversion of municipal solid waste (MSW) by bioconversion of its paper content to ethanol. Ethanol is an Alternative Fuel (ISSUE: B-11) and the outcome of this project is expected to define the Best Process for Industrial Production of Ethanol from Ontario Renewable Resources (TOPIC: B-11.2). Waste paper generation in Ontario is about 3.5 million metric tons per annum, 35 - 40% of MSW. The amount of paper presently diverted to recycling is quite small, and planned increases are not likely to exceed 10% of the total generated. Waste newspaper collection in the Blue Box program has a higher rate of recycling; however, this is still not a high proportion of total paper, which includes, as well as newspaper, also paperboard packaging, Industrial, Commercial and Institutional (I.C.I.) papers, food cartons, magazines and other household papers as described in Section 3 below. Efforts to reduce the amount of paper going to landfill are urgent.

This Project is directed to reducing the volume of MSW now going to landfill by diversion of its paper content and its bioconversion to a marketable product, the environmentally friendly, non-petroleum, high octane motor fuel ethanol.

In our Project Proposal, extracts of which are in Appendix A-1, we proposed to apply to MSW paper our extensive experience in the bioconversion of cellulose, the major component of paper. Our process begins with the enzymatic saccharification of the cellulose in waste paper, followed by fermentation of the resulting sugars to ethanol.

Our proposal included study of a process for in-plant low-cost enzyme production, the target being to bring the cost of cellulase enzymes to the same relative level as commercial starch-saccharifying enzymes. This is necessary because the present costs of cellulose-saccharifying enzymes are much higher than starch-saccharifying enzymes for the same effectiveness. The present Final Report describes in detail our researches on enzyme production. However, enzyme research was not financed by the Ministry of Environment, but by the Natural Sciences and Engineering Research Council of Canada. It is included here because low-cost enzyme is an essential component of a viable industrial process for bioconversion of waste paper to ethanol.

Our proposal listed these specific objectives of our research:

1. To obtain a representative sample of the light fraction of mechanically separated MSW and by analysis determine its potential for saccharification and fermentation.
2. To saccharify the paper component of separated MSW (MSW-paper quantitatively, to measure the volume reduction and to make a mass balance of the process.
3. To convert MSW-paper to ethanol by the best process and to measure yields and enzyme requirements.
4. To study processes for on-site manufacture of cellulase enzymes.
5. To prepare a report which summarizes the results of this work, and considers the economic and technical factors in scale-up.

The present Final Report responds to Objective 5 precisely, by summarizing the results of the work, and examining the economics of these processes quite carefully. We have made considerable progress in each of the areas specified, we are able to define a best process for bioconversion of waste paper in sufficient detail to give us confidence to proceed to larger scale biofuel production (see Section 12, Technology Transfer).

2. Background and Literature Review

2.1 Background

A recent issue of the *OECD Observer*, the bimonthly publication of the (European) Organization for Economic Cooperation and Development, reported on "The Mounting Pile of Waste Paper" in which they asked "Can you imagine a pile of 150 million tons of waste paper? That is approximately the quantity the OECD countries generated in 1990. Compressed into bales, it would cover the entire city of Paris to a depth of two and one-half meters" (Figure 1). This amount, 150 million (metric) tons of waste paper annually, refers to the most advanced countries of Europe, and the measures taken to cope with it are inadequate. About 60% of it ends up in landfill. Landfill sites are becoming scarce in many OECD countries and the costs of operating them are increasing. Domestic paper industries absorb about 30% of the waste paper, while incineration, which is now banned in Ontario, takes care of the rest. Where waste paper cannot be recycled, incineration, when coupled with energy recovery, is being increasingly considered as an alternative to landfill, an option not open to us in Ontario.

This recital can be applied to Ontario except that there is less paper recovery and very little incineration. Recent measures have increased pressures for recycling waste paper, and new de-inking and production facilities are either on-stream (Atlantic Packaging in Whitby) or coming into production in the next few years. These apply particularly to newsprint. The increase in recycling has been stimulated in part by the Blue Box program, in part by new US regulations mandating recycled paper content in government documents. Metro Toronto has added to this pressure by, nominally, refusing to accept I.C.I. papers, for example, at landfill sites. This has resulted in a large haul of Ontario waste paper, and other solid

The Mounting Pile of Waste Paper

4

Wilhelm Kurth

Can you imagine a pile of 150 million tons of waste paper? That is approximately the quantity the OECD countries generated in 1990, the largest amount ever. Compressed into bales, it would cover the entire city of Paris to a depth of two and a half metres. What measures are being adopted to deal with this growing problem?[1]

The waste paper generated in the OECD area is made up of a mynad of paper and board products – newspapers, magazines, packaging material, office documentation, private and business correspondence, advertising material, kitchen paper towels, and many others. And, with the growing consumption of paper and board products in advanced economies, the amount gets higher and higher. Most paper ends up as solid waste immediately after serving its purpose; other forms escape that fate – for some time, at least – because their use is more permanent: books, permanent records, wallpapers, and a number of industrial uses (for example, in shoes and cars, or as building and insulation material). If these products were added to the pile, it would be some 20% higher.

How do societies cope with the waste-paper pile? The Figure depicts the principal means the OECD countries use to dispose of waste paper. In almost all of them, the most important method is the traditional one of landfilling (that is, controlled disposal on land): up to 63% of all waste paper ends up here. But landfill sites are growing scarcer in many OECD countries, and the costs of operating them are increasing.

The second biggest outlet is recycling in the domestic paper industries themselves, which in OECD countries absorb

1. **Environmental Issues: Waste Paper**, available free of charge from the Sectoral Issues Division of the OECD Directorate for Science, Technology and Industry.

Wilhelm Kurth works in the Sectoral Issues Division of the OECD Directorate for Science, Technology and Industry.

Figure 1.

wastes, to US landfill sites. Such diversion, which was not the intent of the regulations, has slightly relieved pressure to locate new landfill sites for Metro garbage, but at the same time has resulted in an amazingly large loss of Metro income in the form of lost tipping fees, estimated at $100 million in 1991.

There are limits to present programs for recycling paper. There are about 12 paper mills in Canada equipped or equipping themselves for recycling waste paper. Atlantic Packaging, in Whitby, Ontario, has a design capacity of 150,000 tons per year for newsprint from waste newspaper. Both the Crofton and Port Alberni paper mills on Vancouver Island are incorporating recycled pulp at the rate of about 40% of their furnish. At 40% recycled content, these mills believe they have much more control over the quality of their final product. Using recycled fibers can lead to process problems. Build-up of residual ink and sticky matter on the paper machines leads to lost time from frequent washups and downgraded production. Inter City Papers, Mississauga, "Closes the Loop" by supplying recycled paper products made from the customers' own waste paper. Stake Technologies Ltd., Norval, Ontario, has applied its "steam explosion" process to the recycling of lower grades of waste paper more efficiently.

The continuing disposal of MSW in landfill is generating political difficulties in Ontario municipalities and is increasingly expensive as MSW is hauled over greater distances. The expected life of many major landfill sites is nearing an end. Any significant reduction in the volume of MSW would extend their life, while the conversion of as much as possible of MSW to marketable products would help relieve its present economic burden. We estimate that, carried to its full potential, bioconversion of MSW paper to ethanol would reduce MSW volume by 40% to 50 %. The technology employed is multi-media, in reducing the volume of solid waste and at the same time providing air

pollution reduction by the use of a clean-burning alternative motor fuel ethanol; it is **sustainable** in that waste paper feedstock will continue to be available for the foreseeable future; and it is a **marketable** and **exportable** technology (see Section 12 Technology Transfer and Entrepreneurial Prospects).

The use of petroleum-based motor fuels has been a large factor in the creation of smog over Toronto and other Ontario cities, and a major contributor to the threatening greenhouse effect. Extensive experience in the US, Brazil and elsewhere has demonstrated that the use of gasohol, an ethanol-extended (or substituted) gasoline has provided substantial relief from these environmentally harmful effects. Colorado and other US states have mandated the use of gasohol when smog is most severe. The government of Manitoba has announced that it plans to use gasohol in its fleet of 2800 vehicles. We can expect that their experience will stimulate similar action here in Ontario. An excellent review of the contribution of auto emissions to air pollution "Motor Vehicles and Air Pollution" Alan Goodall, *Canadian Social Trends,* Statistics Canada, Spring 1992 pp 21-26 (see Appendix A-2), begins

"Motor vehicles in general, and automobiles in particular, continue to be a major source of air pollution in Canada. Although emissions have been regulated since the early 1970s, motor vehicles remain the largest source of several pollutants that adversely affect human health and the environment. Most Canadians, particularly those in urban areas, continue to be exposed to potentially harmful levels of these pollutants. Ozone levels, for example, exceeded maximum acceptable levels within the Windsor-Quebec City corridor, home to most Canadians" (and most Ontarians) "more often than in other Canadian regions. In 1988 one-hour concentrations exceeding acceptable limits were recorded 189 times in Windsor, 157 in North York, 149 in London and 112 in Oakville. During high

concentrations, those most at risk include people with asthma or lung disease, children, the elderly, and individuals who work or excercise heavily outdoors. While other emission products also harm vegetation, ground level ozone in particular is known to adversely affect crops, trees, vegetables and ornamental plants. . . Depending on the frequency of high ozone episodes, the combined commercial crop losses can range from $17 - 70 million per year."

Goodall's hope for improvement lies in better maintenance of emission control equipment on motor vehicles, and on more use of public transportation. New British Columbia clean air regulations require annual testing and maintenance of emission control equipment on automobiles. Goodall also provides a useful list of references, mostly from Environment Canada.

Potential markets for ethanol in Ontario are large, annual motor fuel consumption being about 12,000 million liters. If that were substituted by ethanol to the extent of 10%, the market for that use would be 1,200 million liters per year. Ontario MSW paper, estimated at 3.5 million tons per year, is capable of providing on bioconversion 1,200 million liters per year, just enough to supply fully a 10% ethanol substituted gasohol. In so doing, it would divert more than 2 million tons of MSW per year from landfill. It would require 12 - 24 full size waste paper-based ethanol plants employing about 2,500 workers permanently on site plus an equal number occupied in the supply of raw materials, and about 20,000 man years of employment in construction over 5 years. Investment of about $60 million in each plant would amount to $1.2 - 1.5 billion. The promise of such enormous industrial activity and environmental benefit demands serious attention.

These benefits have long been recognized. Two major handicaps which have delayed the widespread installation of facilities for bioconversion of MSW to fuel ethanol are an unfavourable political

climate and uncertainty about the availability of economically viable technology. The hesitant political climate arises from skepticism by Canadian decision-makers that environmental considerations belong near the top of an already crowded agenda, from their doubts that renewable resources could supply the huge volumes required to satisfy our motor fuel markets, and in part from the powerful political influence of entrenched fuel suppliers and landfill stakeholders. Economics has in fact played very little role in avoiding renewable biofuels, or in developing other fuel supplies as witness the billions of dollars poured into economic and technical sinkholes in the Arctic, in tar sands and so-called heavy oils, and in the Gulf war. The money so spent could have provided Canadian (and American) needs for gasohol many times over, and those resource allocations are quite adequate to establish biofuels such as ethanol on a firm basis.

The technical uncertainty is a hangover from the large research effort which has been devoted to the bioconversion of wood to ethanol, an effort to which the author was a major contributor, without yielding a favourable outcome in the form of a wood-based ethanol plant in Ontario. There are many reasons why wood-based ethanol has not yet found a place in Ontario, but these are largely irrelevant since the production of ethanol from paper avoids many of the complexities of wood as a feedstock, and is a much simpler process. The paper to ethanol process is precisely similar to the starch to alcohol process, long established as a huge industry. For alcohol production from starch, the feedstocks are corn, barley, wheat and other grains. The starch is saccharified by enzymes and the resulting sugars are fermented by yeast to alcohol. In production of the alcohol ethanol from paper, the same process is used: enzymatic saccharification and yeast fermentation. The major differences arise from the lack of experience over time and scale in the manufacture of cellulase (paper) enzymes compare to starch enzymes. Current market prices for cellulase enzymes are about 10 times those for starch enzymes for equal effectiveness. This is the

major, and perhaps the only major, technical handicap which needs serious attention to cross the economic threshold. Yet processes for making the two classes of enzyme are very similar indeed, and in this era of biotechnology the costs can without question be brought into line. This is a principal area of study in our laboratory, and as can be seen from Section 6 Enzyme Supply and from Appendices A-6, A-7 and A-8, we now have an excellent process for the most important paper liquefying enzyme.

2.2 Literature Review

The literature on cellulose saccharification by enzymes can be said to begin with the studies carried out in the 1950s at the U.S. Army Research Laboratory, Natick, Massachusetts, by Elwyn T. Reese, Mary Mandels and their colleagues. The degradation of canvas harness in troop clothing in the South Pacific was traced to a green mould *Trichoderma viride,* which produced the cellulose-saccharifying enzyme cellulase. A pilot plant was built at Natick to convert cellulosic feedstocks, including waste paper, to fermentable sugars. This research falls essentially into two groups: the production and nature of the cellulases (the cellulase complex), and the saccharification of cellulose by cellulase. The research on production of cellulase is described by Ryu and Mandels, *Enzyme and Microbial Technology 2: 91-102* (1980); also by M.J. Somogyi, a famous sugar chemist, in *J. Biological Chemistry 195:* 19-23 (1952), and in the book *Cellulases and Their Applications, eds.* G.J. Hajny and E.T. Reese, American Chemical Society 1969, which contains a chapter by Mary Mandels "The Production of Cellulase." Other useful early papers on the production of cellulase are from the laboratory of T.K. Ghose (A.N. Pathak and T.K. Ghose "Cellulases I, Sources and Technology," *Process Biochemistry 8,* 35-38 (1973), and "Cellulases II, Applications," *Process Biochemistry 8,* 20-21, 24 (1973); and the major paper by M.P. Coughlan, "The Properties of Fungal and Bacterial Cellulases, with Comment on Their Production and Application," *Biotechnology and Genetic Engineering Reviews 3:* 39-109 (1985).

New insight into the isoenzyme components of the cellulase complex has recently been provided by T.-M. Enari and M.-L. Niku-Paavola in the paper "Enzymatic Hydrolysis of Cellulose: is the Current Theory of the Mechanism of Hydrolysis Valid?" *CRC Critical Reviews in Biotechnology 5,* 67-87 (1987).

The recent book "Enzymes in Biomass Conversion" eds. G.F. Leatham and M.E. Himmel, A.C.S. Symposium Series 460, Washington 1991, contains 38 papers on this subject. Two somewhat earlier books related to the use of cellulases for cellulose sacchharification are "Enzymatic Conversion of Cellulosic Materials: Technology and Applications," E.J. Gaden, M.H. Mandels, E.T. Reese and L.A. Spano,_*"Biotechnoiogy and Bioengineering Symposium 6"* Interscience 1976; and "Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis," eds. R.D. Brown Jr. and L. Jurasek, A.C.S. 1979. In honour of the contributions to this subject made by Elwyn T. Reese, the microorganism *Trichoderma viride* has been renamed *Trichoderma reesei.*

A recent paper by Japanese investigators appears as Chapter 18 in "Cellulose: Structure, Modification and Hydrolysis," eds. R.A. Young and R.M. Rowell, Wiley 1986 entitled "Continuous Pretreatment and Enzymatic Saccharification of Lignocellulosics," by S. Moriyama and T. Saida. The same book also has a chapter which reviews acid hydrolysis of cellulose "Comparative Effectiveness of Various Acids for Hydrolysis of Cellulosics" by M. Wayman. There are a great many centers of research on lignocellulosics in Japan which aim to produce ethanol. Professors T, Sawada, at the University of Kanazawa, and M. Tanahashi of Gifu University, have been prolific publishers, and recently each sent us a collection of his many papers on cellulosic pretreatment and hydrolysis. NEDO, the New Energy and Industrial Technology Development Organization, Tokyo, publishes an Annual Research and Development Report which abstracts public and private sector research and development in this field in English.

In 1982 D.S. Chahal produced a monumental review of the subject "Enzymatic Hydrolysis of Cellulose: State of the Art" for the National Research Council of Canada, Report NRC 20283. Chahal has continued to publish on this subject, most recently a chapter in the book "Enzymes in Biomass Conversion" mentioned above.

European interest in MSW-based biofuel production is reflected in a number of research papers, among which we can mention H. Esterbauer, W. Steiner and their colleagues "Production of *Trichoderma* Cellulase in Laboratory and Pilot Scale," *Bioresource Technology 35*, 51 - 65 (1991); and I. Persson, F. Tjerneld and B. Hahn-Hagerdal "Fungal Cellulolytic Enzyme Production: a Review," *Process Biochemistry 26,* 65- 74 (1991). A short book "Verwertung von Biomasse," F.J. Reitter and M. Reichert, Muller Karlsruhe 1984 describes the BIOL process developed by the Munich group Zellplan. This has been updated in a recent manuscript. The large pilot plant for bioconversion of cellulosics to ethanol (or butanol) at Soustons, in southern France, built by I.F.P. (Institut Français du Pétrole) has been described in some detail in a recent manuscript in press. I.F.P. are associated with Stake Technology of Norval, Ontario, which has recently published a description of their process for saccharification of aspen wood, *Bioresource Technology 35*, 23 - 32 (1991).

Active groups in the US include TVA, which is still pursuing dilute acid hydrolysis of cellulosics, and have recently published several papers on MSW hydrolysis and fermentation. They presented their results at a Symposium on Biotechnology for Fuels and Chemicals in Gatlinburg, Tennessee, May 11 - 15, 1992. Both Prof. Hans Grethlein, of Michigan Biotechnology Institute, and C.D. Scott, of Oak Ridge, presented their researches on ethanol production from MSW at the same Symposium. The former Solar Energy Research Institute (SERI), now the National Renewable Energy Laboratory (NREL), has appointed Ralph Overend, of our National Research Council, to head up their research on biofuels, but their new direction is unclear.

Professor L.O.N. Ingram, of the University of Florida, Gainesville, has applied genetic engineering to this problem, and has succeeded in placing cellulase enzymes in *E. coli,* a common gut bacterium, which is also a xylose fermenter. Several laboratories have been studying his new bacterium, with mixed success.

A recent review of British publications in the area of bioconversion of biomass "Concerted Action on Enzymatic Hydrolysis" revealed little of substance. Perhaps the major work is at the University of Swansea on bacterial cellulases. At University College Dublin, G. Halliwell is studying high temperature fermentation. The review also mentions research on MSW by A. Mottet at the Faculte d'Agronomie in Gembloux, Belgium, and on continuous hydrolysis and subsequent fermentation by G. Geiseler, Friedrichshafen, Germany.

Our laboratory in the University of Toronto has published several papers in this field. The M.A.Sc. thesis of Wancheng Zhao "Production of Cellulase by *Trichoderma reesei* Rut C-30 Induced by Aspen Cellodextrin" was published in 1988. A 1989 paper "Novel Immobilized Enzyme and Yeast Continuous Fermentation System", Shu Chen and Morris Wayman, *Process Biochemistry 24,* 204 - 207 introduced some interesting concepts. More recent papers from our laboratory, included here as Appendices A-3 - A-8, are: "Bioconversion of Waste Paper to Ethanol," "Pretreatment of Waste Paper in a Vapour Cracker and Bioconversion of the Pretreated Fiber to Ethanol," "Bioconversion of Refuse Derived Fuel to Ethanol," "Cellulase Production Induced by Carbon Sources Derived from Waste Paper," "Novel Inducers Derived from Starch for Cellulase Production by *Trichoderma reesei,"* " Cellulase Production by *Trichoderma reesei* using Whole Wheat Flour as a Carbon Source."

Our recent book "Biotechnology of Biomass Conversion: Fuels and Chemicals From Renewable Resources," Morris Wayman and

Sarad Parekh, John Wiley & Sons Ltd UK and Prentice Hall US 1990 is an excellent review which places ethanol production from grain, tubers and cellulosics in context. Unfortunately the book does not treat MSW as a resource adequately; discussions for its translation into German are now proceeding, and the German edition may have a more extensive discussion of that subject.

This literature review suggests that there is a diversity of approaches to pretreatment, saccharification and fermentation of cellulosics, including MSW-paper and RDF. These papers must represent our current concept of "the state of the art" until such time as pilot plants, demonstration plants and full scale bioconversion plants are built and operated. This may be quite soon (see Section 12 Technology Transfer and Entrepreneurial Prospects).

3. Waste Paper

3.1 Availability

There are no reliable hard data on the amount of waste paper generated in Ontario, or even on the percentage of paper in MSW. In part, this is because such figures vary over place and time. We have assumed total MSW generated in Ontario to be about 1 ton per person per year, that is about 10 million tons annually, and that 35 to 40% of MSW is of paper origin. We are only certain that the total of waste paper generated is a very large quantity (compare OECD data, 150 million tons annually, Figure 1). In this Report, "ton" means metric ton.

Also we have no good data on the percentage of various kinds of paper within MSW-paper. MSW-paper contains newspaper, office papers white or coloured, corrugated paper, food cartons such as milk cartons, other forms of paperboard packaging such as cereal boxes, kraft (brown) wrapping paper, magazines, remaindered paperback books and others of the 1,000 varieties of paper currently in production. In our research we have made certain assumptions about the composition of MSW-paper; in studying a synthetic mixed MSW-paper we have used the following composition:

25% office paper
25% newspaper
20% kraft paper
20% cartons, including both food cartons and paperboard packaging
10% corrugated carton
100%

This mix of papers in MSW is based on a small amount of sampling and picking apart, not really enough to be statistically significant; however, it is scarcely worthwhile to go to much trouble to have more accurate data because the composition of MSW-paper is changing rather rapidly. In terms of waste paper availability, there are at least four types of waste paper where recycling has, or can have, a large impact: corrugated boxes, newspapers, I.C.I. (Industrial, Commercial and Institutional) waste paper, in which segregaation and/or recycling now plays a significant role, and paperboard packaging, for which segregation is planned. Corrugated boxes have long been recycled in Ontario, there being large paper mills which use primarily used corrugated boxes of which Strathcona Paper, Napanee, and Trent Valley Paperboard Industries, Trenton, are perhaps best known. In recent years, supermarkets, shopping malls and even small stores have segregated and baled corrugated boxes for recycling. Old newspapers now also fall in a separate category, since old newspapers are being recycled into fresh newsprint in several large paper mills, of which Atlantic Packaging, Whitby, is the largest in operation. Quebec and Ontario Paper, Thorold, may use even more recycled newsprint when its conversion is complete. (As a historical note, for many years the old Ontario Paper Company, now Quebec and Ontario Paper Company, blended about 3% of old newspapers into its regular newsprint production without any deinking, with no adverse effects on quality. In the U.S. there are several very large newsprint mills which use 100% old newspapers as feedstock, beginning 30 years ago with Garden State Papermill, near New York City). Since 90% of newsprint made in Canada is exported, domestic old newspapers cannot supply enough paper for many recycled

newsprint mills, and the industry faces a dilemma in its attempt to meet U.S. regulations. In our forward planning, we cannot count on much, or any, old newspapers being available for bioconversion to ethanol. The Blue Box program has performed excellently in segregating and gathering old newspapers for recycling, and this cannot be considered as a feedstock for the ethanol development.

I.C.I. paper falls in a different category. When Metro Toronto announced "We Cannot Take It Any More," a new situation developed where I.C.I. waste papers are segregated but only a small proportion are recycled into fresh fine papers. Much of this I.C.I. paper is generally available at very low cost, zero cost or even negative cost. Because of its favourable composition compared to paper recovered mechanically from total mixed MSW, bioconversion of waste office paper to ethanol is a simpler process with few complications, as described in the next Section 4. We use it as a model in developing and improving our bioconversion process, and we expect that both in the short run and in the long run enough I.C.I. paper will be available to supply the first full scale bioconversion plants.

The Paperboard Packaging Environmental Council is considering means of reducing the flow of old cereal boxes etc. to landfill. It is planned to pilot segregation at source of waste paperboard in an Ontario community, perhaps as an addition to the Blue Box program, much as old newspapers are now segregated and collected. This offers the prospect of reduced landfilling of paperboard, and also of making some tonnage available for recycling into fresh paper, or for bioconversion to ethanol.

3.2 The Paper-Enriched Fraction of MSW

The separation of paper or a paper-enriched fraction from MSW is widely practiced in Europe but not in Ontario. Installations for mechanical separation of a paper-enriched fraction are complex and expensive, and are not operated primarily for paper-enrichment but for calorie enrichment, that is the separation of a fraction of MSW with relatively high BTU content, to be used as a fuel, Refuse Derived Fuel (RDF), to be burned in large boilers. The products of combustion are used to produce steam, generate electricity and provide district heat. Enrichment is ordinarily effected by some combination of attrition mills, screens and air classifiers. The fraction of RDF which is enriched in paper is referred to also as the light fraction. While the input to such a plant may be 30 - 40% paper, the light fraction, which is the fraction of interest to us, is 50 - 55% paper. In Figures 2 and 3 we illustrate two MSW separation systems well established in industrial use. Figure 2 illustrates the BRINI system, developed and operating on a full scale, which accepts MSW from towns in Sweden such as Kovik near Stockholm and provides fuel for steam generation. In Figure 2, at **A** MSW is delivered to the plant, where it is conveyed from a storage bin to a pulverizer **B**. About 80% of the MSW is reduced in the mill to particles 50 mm in diameter. A classifier **C** separates a light fraction (RDF), a heavy fraction mostly metals and a compostable fraction. The heavy fraction **D** is primarily used as scrap in the metallurgical industries, while the compostables fraction **E** is fed to a mobile composter. The light fraction **F** consists mainly of paper (50 - 70%) and plastic film (10%). It is dried **G** to about 15% moisture and pelletized **H**. The light fraction comprises 50 - 70% of the total volume of the MSW. It has an energy content of 7,300 BTU/lb (17 MJ/kg) of pellets. The Kovik plant processes 40,000 tons of MSW annually, and it is claimed that 90% of the MSW is converted to valuable products and only a small fraction remains as residual waste.

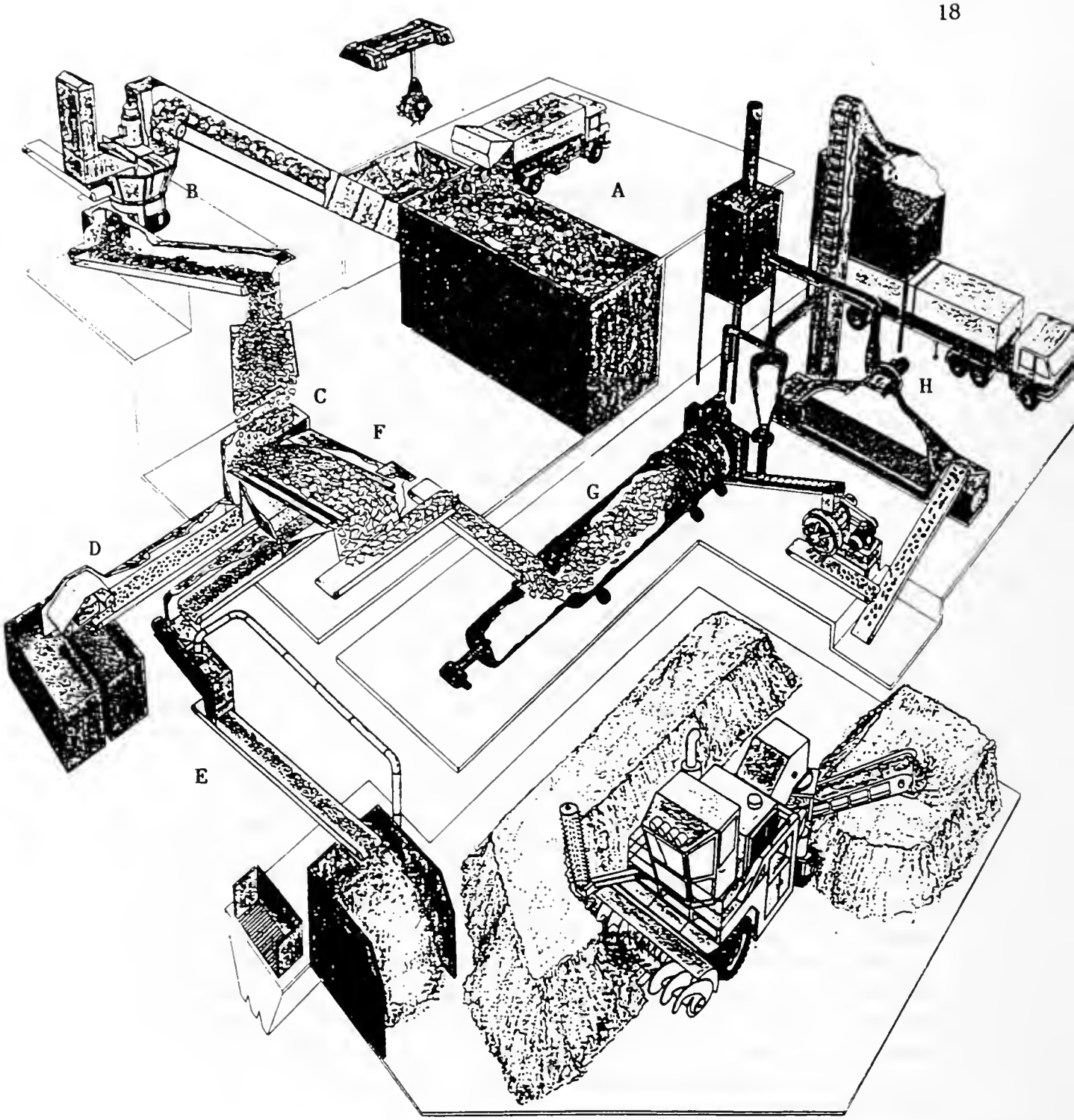

Figure 2: A TOTAL WASTE-RECOVERY SYSTEM.
THE BRINI FRACTIONATION SYSTEM FOR MUNICIPAL SOLID WASTE

The Lundell waste separation system is, in principle, simpler, as seen in Figure 3. It is a linear system, built in modules each of which accepts 100 tons/day MSW. Like the BRINI system, it claims to turn 90% of MSW into valuable products. Separation of fractions is accomplished by a series of air-assisted screens. Following shredding, plastic is screened out (1 - 5% of the total) then a heavy fraction, mainly steel (5 - 10%). Compostables make up 20 - 30% of the product, and in the Lundell system, aluminum is separated and recovered ahead of the light fraction or energy fraction. The light fraction is 50 - 60% of the MSW fed into the plant, and in Figure 3 it is shown going through a pelletizer.

The two MSW fractionation systems have much in common and claim to achieve about the same results. Cost figures provided to us suggest that the BRINI system is much more expensive. However, that should be taken as tentative, since no firm quotations are available. A plan was put forward to install a BRINI system in Nanaimo, B.C., but the B.C. government backed out, perhaps because of the high cost. North American rights to the BRINI system ae held by Waste Management of North America, Oak Brook, Illinois, but there are no BRINI plants operating in North America. The B.C. company promoting the Nanaimo project was AEC Advanced Ecology Corporation, of Vancouver.

The Lundell System is represented by Lundell Canada Inc., of Chatham, Ontario. The Lundell company lists 10 plants in operation in the U.S. in 1990, with 3 others under construction and several others under negotiation, including plants in Italy and Australia. All of the existing Lundell plants in 1990 were 100 - 125 tons MSW/day, while the plants under negotiation included much larger installations, 500 - 1,000 tons MSW/day. It is assumed that the larger plants are an assemblage of 100 ton/day modules.

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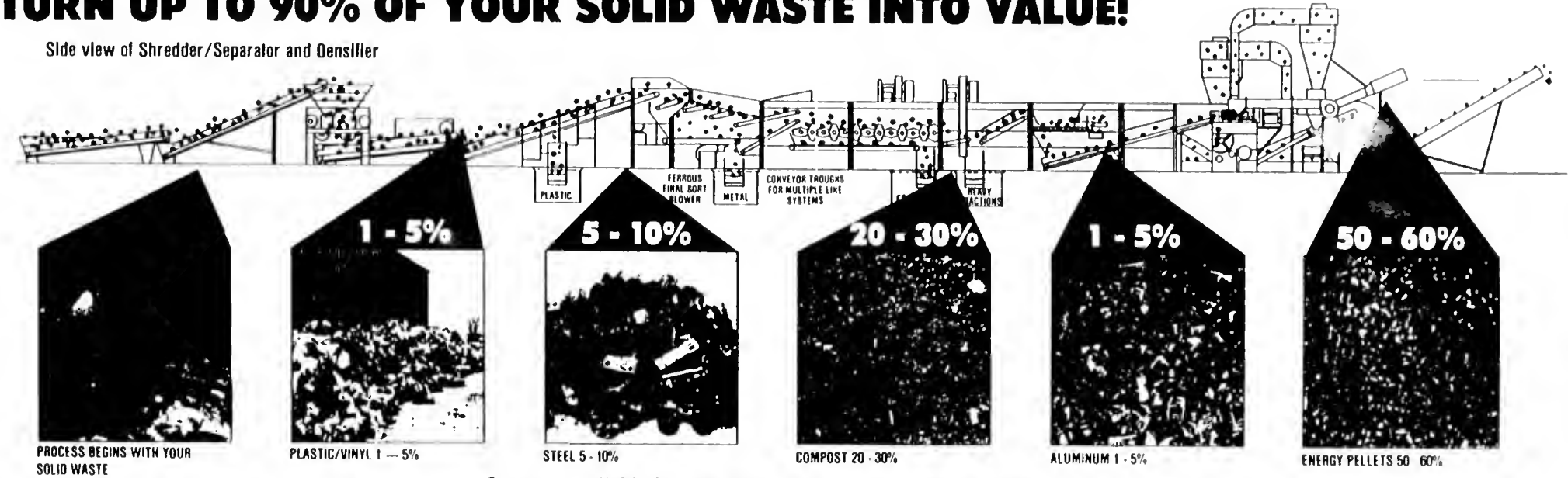
9 PATENTS WITH OVER 169 PATENT CLAIMS IN PROCESS ON THESE DENSIFYING AND SEPARATING SYSTEMS

TURN UP TO 90% OF YOUR SOLID WASTE INTO VALUE!

Side view of Shredder/Separator and Densifier



PROCESS BEGINS WITH YOUR SOLID WASTE

PLASTIC/VINYL 1 — 5%

STEEL 5 - 10%

COMPOST 20 - 30%

ALUMINUM 1 - 5%

ENERGY PELLETS 50 - 60%

Systems available for baling paper or producing energy pellets
Available in sizes to handle from 50 TPD to 5000 tons per day

FIGURE 3. The Lundell System of Fractionation of Municipal Solid Waste

Claims are made for higher paper or BTU content, but we have not seen samples of higher paper RDF. An MSW fractionation system to raise paper content still higher can be built, but the cost and performance (yields) of such systems have not been estimated. RDF, like other papers, is a good source of ethanol, but of course cannot be expected to yield as much ethanol/ton since its cellulose content is relatively low compared to any of the other papers tested. Also there are concerns about the effects of contaminants on fermentability. We have not observed such effects, except perhaps somewhat slower fermentation (see Appendix A-4). However, we have concluded that the design of a facility for bioconversion of RDF to ethanol would take such concerns into consideration aand should provide for them, by a pretreatment step (see Section 5 and Appendix A-4). Among other virtues, pretreatment such as that described in Appendix A-4 would ensure sterilization of the feedstock and freedom from microbial contamination.

3.3 Analysis of Waste Papers

The following Tables present our analyses of various kinds of paper found in MSW and of RDF, as well as their ethanol potential based on cellulose content.

Table 1. Waste Papers, Summative Analysis and Ethanol Potential

	Office Paper	News Paper	Kraft Paper	Milk Carton	Corrugated Carton	Paper-Board	Mixed WastePaper*
moisture, %	6.6	8.9	5.5	5.9	6.0	6.8	6.3
ash, %	9.1	0.3	1.3	0.1	1.1	NA	2.8
carbohydrates as sugars,%	88.0	70.9	81.3	77.2	69.1	77.6	78.3
ethanol potential, liters/ton**	555	445	512	485	433	488	492

* mixed waste paper: office paper, 25%; newspaper, 25%; kraft paper, 20%; milk carton, 20%; corrugated caton, 10%.

**ethanol potential =

$$\frac{(\%\ \text{carbohydrate} - 2^{***}) \times 0.51 \times 10}{0.79}$$

*** 2% is subtracted from carbohydrates as sugars to provide for the presence of arabinose, which is not fermentable

ethanol potential of pure cellulose = 715 liters/ton

ton in this paper is always metric ton

Table 2. Refuse Derived Fuel: Summative Analysis and Ethanol Potential

		Lundell			WMI	
	BRINI	Pellets	Shredded	NEI	Pellets	Shredded
moisture, %	9.9	8.3	5.9	5.8	6.0	6.4
ash, %	15.2	7.3	3.8	10.3	13.9	9.0
carbohydrates as sugars, %	69.5	58.9	58.1	54.7	53.5	59.5
ethanol potential liters/ton	435	367	362	340	330	370

NEI is National Ecology Inc., a subsidiary of Babcock & Wilcox, operates an energy facility in West Palm Beach, Florida, which processes 2700 tons per day of MSW to produce steam and electricity

WMI, Waste Management of North America, Inc., is one of the largest collectors of MSW in North America, and has the North American rights to the BRINI process

Concern has been expressed about the contaminants which must be present in RDF. The following Table 3 lists contaminants found in 2 samples of RDF from different sources.

Table 3. Contaminants in RDF

	Lundell	NEI
Paper*	+ +	+ +
Plastics	+ +	+ +
Aluminum foil	+ +	+ +
Wood	-	+
Cloth	-	+ +
Styrofoam	+	+
Sand, dirt	+ +	+ +
Metal	-	+
Hair	-	+

* These samples of RDF contained newspaper, white bond, computer paper, boxboard, corrugated, kraft, and coated wrapping papers. The NEI sample contained tissue also.

Paperboard packaging refers to cereal boxes and the like, which form a significant percentage of the paper in MSW. A sample of paperboard said to be representative was obtained from The Paperboard Packaging Environmental Council (PPEC). Its composition was determined as follows:

Table 4. Waste paperboard packaging composition

Moisture, %	6.8	
Acid insolubles (lignin + ash)	16.4	
Polysaccharides, %	71.3	= 78.4% sugars
made up of		
glucan	50.1	
cellobiose	2.2	
xylan	12.2	
galactan	1.3	
arabinan + mannan	5.5	

ethanol potential, 493 liters/ton

3.4 Potential for Ethanol Production

The results of analysis of this representative sample of waste paperboard packaging as shown in Table 4 agree rather well with the results shown for paperboard in Table 1, which latter was a sample gathered from domestic sources by the laboratory. The carbohydrate composition shown here indicates a high percentage of xylose, which is derived from hardwood fiber suggesting the use of such species as poplar and birch in making paperboard, whereas newsprint, kraft and most of the other papers in MSW are made mainly from such conifers as spruce, balsam and pine. An industrial facility for bioconversion of paperboard to ethanol would be well advised to use xylose-fermenting yeasts and conditions conducive to xylose fermentation in order to obtain high yields of ethanol.

The potential production of ethanol from waste paper, refuse derived fuel and paperboard as shown in Tables 1, 2 and 4 indicates a range of 433 to 555 liters/ton for the papers, with paperboard falling within this range, and 330 to 435 for the RDF. Among the papers, ethanol yield from office paper is significantly higher than the others, while corrugated carton and newspaper is significantly lower. A ranking is as follows: office paper (bond) > kraft > paperboard > milk carton > newspaper >corrugated carton. If we exclude newspaper and corrugated carton from the presumed available feedstocks, the range of the others is rather narrow: 485 - 555 liters/ton, a difference of 14%.

The ethanol potential of the samples of RDF reported in Table 2 shows considerable variability, from 330 to 435 liters/ton, a difference of 32%. The best of these was the BRINI sample. The other 5 samples fell in a much narrower range, the difference between high and low being 12%. Of these 5, the highest and lowest samples both came from the same source, WMI. It is apparent that we can expect significant variability in composition and in ethanol potential with feedstocks of waste paper, RDF or any other fraction recovered from MSW. By arrangement of storage bins of large capacity and other devices, such variability can to some extent be smoothed out.

4. Bioconversion of Waste Paper to Ethanol

4.1 Process Development Background

The amount of waste paper generated annually in North America and finding its way to landfill sites as MSW is about 100 million tons, enough upon bioconversion to ethanol to replace about 16% of all North American gasoline. The prospect of conversion of so much MSW-paper to fuel ethanol has resulted in considerable pilot plant and research activity. We will here mention 2 large pilot plants

in the U.S. and 1 in France employing our preferred process, enzymatic saccharification and yeast fermentation. In 1976, a facility to convert the cellulose content of MSW to ethanol was installed at the Gulf Chemicals Pittsburgh, Kansas, petrochemical complex. It had a nominal capacity of 1 ton of feedstock/day, enough to produce 80,000 liters of ethanol/year. The process employed cellulase enzymes produced on site by the fungus T. reesei, saccharification being followed by fermentation by common bakers' yeast. In 1980 the feedstock was changed to steam-exploded aspen wood based on the prospect of dedicated plantations, supplied by Iotech. The operation, which was discontinued for lack of funding in 1982, has been described by Emert and Katzen, Chemtech, 610 - 614 (1980).

In 1985, Procter and Gamble Paper Products pulp-paper mill at Mehoopany, Pennsylvania, faced a problem with excessive fibers in their effluents, and established a pilot plant for bioconversion of the cellulosic fibers to ethanol, at the same time considering MSW as a possible feedstock. The pilot plant took in 3 to 5 tons of fibers/day. There was no pretreatment before saccharification, since the cellulose had already had extensive "pretreatment" in the pulping. Simultaneous saccharification and fermentation was practiced, using purchased cellulase enzymes. After about 1 year of operation, the project was abandoned, contributing factors to the pilot plant closure being the high cost of purchased enzymes, and the expected high capital cost for a complete cellulosic ethanol plant.

A pilot plant for the bioconversion of cellulosics to ethanol (or butanol) was built by the Institut Français du Pétrole at Soustons, France, using Stake steam pretreatment followed by saccharification by cellulase enzymes made on site, followed by fermentation. This pilot plant can process 3 tons/hour of cellulosics, including, presumably, MSW. Of these 3, only the Soustons pilot plant is continuing operation, but thus far have employed as feedstocks only woods such as poplar and agricultural residues such as straw, and not waste paper, RDF or MSW.

The definition of the process for bioconversion of MSW-paper to ethanol is a major objective of our research. To develop our process, we first of all adopted office paper as a model, and only after sufficient progress had been made did we study waste paperboard packaging and RDF; and secondly, we adopted performance targets, as follows: 350 - 400 liters of ethanol/ton of waste paper, ethanol concentration 6+% by volume; and 54 hours maximum total time for saccharification and fermentation. In most of our studies we assumed that paper, having been made by defibering wood in a chemical bath at high temperature and pressure, did not require any pretreatment such as steam explosion with or without catalysts; however, in the next section Section 5, we report the results of studies of such pretreatment of waste paper.

The question of the need for or the advantages of pretreatment is one of several we undertook to answer. Other questions bearing upon the best process configuration are: should saccharification be complete before fermentation, or should the two process steps be simultaneous? and if simultaneous, how can the difference between the optimal temperature of saccharification, 45 - 50°C, and fermentation, 28 - 32°C be reconciled? The strategy of feedstock addition is also very important: should the waste paper be added all at once at the beginning, or is fed batch more productive? Finally, can enzymes be made cheaply on site?

This last question is answered more fully in Section 6, Enzyme Supply. In our work, however, until the most recent experiments reported here, we employed purchased enzymes, mainly Multifect S-850 (Finnish Sugar Company, 344 IU/g, 37.9% protein) and Novozym 188 (Novo, 91.5 IU/mL). We should also point out that I.F.P. claims that it now has an excellent process for making cellulase on site, and that Iogen claims that by 1995 their cellulase enzyme will meet our criterion of being equivalent in cost/effectiveness to starch enzymes.

4.2 I.C.I. Waste Paper

None of these samples of waste office paper were given any pretreatment before saccharification. The paper was shredded and cut into squares 1 - 2 cm to a side. Saccharification was carried out in 250 mL shake flasks containing 100 mL saccharification medium, or in 2.5 L fermenters designed for the processing of paper, as described below. The amount of paper was initially 2% to 20% solids. The amount of cellulase enzymes used was 8.6 to 70 IU/g paper, the enzyme being a mixture of 70% cellulase and 30% cellobiase. The pH was adjusted with lime to 4.8. Small amounts of Vitamin B_{12} and trace elements were added, along with a few drops of a surface-active compound Triton X100. Saccharification in the shake flasks was assisted by the addition of several small stainless steel balls. Incubation was at 45°C in a rotary shaker at 150 rpm.

Fermentation was begun either after 6 hours, or when saccharification was apparently complete. In either case the temperature was lowered to 37°C and the yeast added. This temperature was chosen to permit saccharification to continue during fermentation. Nutrients added for fermentation were either YMP (0.3% yeast extract, 0.3% malt extract, 0.5% peptone), or DAP (0.01% diammonium phosphate + 0.02% urea). Initial cell concentrations were 10 - 20 g/L freeze dried weight. Anaerobic closures were used for fermentation.

For somewhat larger scale saccharification and fermentation, conventional stirred tank fermenters proved unsatisfactory, since the paper clogged the internals even at low consistency. A simple, efficient bioreactor for paper processing is shown in Figure 4. The body of the bioreactor is a recycled plastic 2.5 L chemical bottle, held horizontally in a reciprocal shaker water bath with temperature

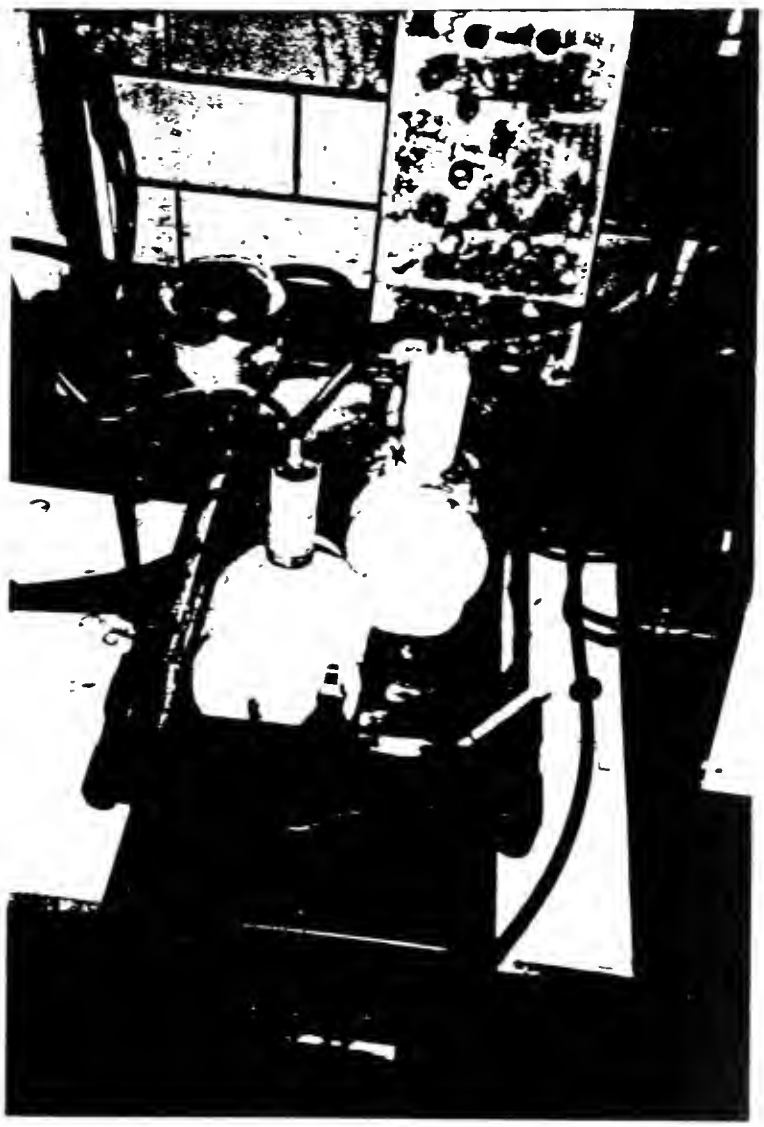


Figure 4. 2.5 liter bioreactor

control. A 100 mL bottle is sealed to a hole in its side, the bioreactor being oriented so that this smaller bottle is vertical, serving to keep the gas outlet on its head free of paper. The gas outlet is equipped with an anaerobic closure. In operation, the bioreactor cycles about 120 times per minute, enough to keep the paper in suspension even at high consistency. For use in aerobic fermentation, such as enzyme synthesis, an air inlet and air flowmeter were added. Usually in this bioreactor 100 g waste paper was processed suspended in 1 L medium, and after partial liquefaction a second 100 g paper was added.

Table 5 reports the results of saccharification (without fermentation) of shredded white office paper (WOP) by a 70:30 mixture of cellulase and cellobiase in 100 mL saccharification medium in 250 mL shake flasks at pH 4.8, incubated at 45°C on a rotary shaker at 150 rpm with the additives named above. With 8.6 IU enzyme/g paper at 5% solids over a period of 72 hours, 69.8% saccharification was obtained. When the ratio of enzyme to paper and the percentage of solids wee both doubled, yields of sugars were 80.5% in 48 hours.

Simultaneous saccharifiction and fermentation of WOP gave the results shown in Table 6. This is not a truly simultaneous saccharification-fermentation: WOP was treated with saccharification medium at 45°C for 6 hours, then cooled to 37°C and yeast and YMP added. In this procedure, to 100 mL enzyme solution containing 240 IU cellulase and 27 IU cellobiase plus the aforementioned additives, 10 g WOP was added, incubated on a rotary shaker at 45°C for 6 hours, then cooled to 37°C and yeast and nutrients added for anaerobic fermentation. After 24 hours, the same amount of WOP and enzyme were added and the mixture incubated for an additional 48 hours. Ethanol was produced at the rate of 353 to 415 liters/ton WOP, at concentrations of 7.1 to 8.3% by volume.

Comparing the results of saccharification without fermentation with those of simultaneous saccharification-fermentation, it is apparent that saccharification proceeds more efficiently in the presence of yeast.

Table 5. Saccharification of Shredded White Office Paper (Without Fermentation)

*E/WOP IU/g	solids %	time hours	total sugars % of WOP	saccharification %
8.6	5	48	57.2	65.0
		72	61.4	69.8
17.2	10	48	70.8	80.5
17.2	**10 + 10	72	47.6	54.1
34.4	10 + 10	72	43.6	49.5
34.4	10 + 10	72	48.8	55.5

*E is 70% cellulase (Multifect S-850) + 30% cellobiase (Novozym 188)

**10 + 10 represents 2 additions of WOP 24 hours apart

Table 6. Simultaneous Saccharification and Fermentation of White Office Paper (WOP)

*E/WOP	time	ETHANOL			
IU/g	hours	g/L	% by volume	L/ton	g/g glucose**
17.2	72	55.8	7.1	353	0.41
17.2	96	59.3	7.5	375	.44
34.4	72	63.0	8.0	395	.47
34.4	96	65.8	8.3	415	.49

*WOP composition, in %: moisture 6.10; acid insolubles 10.0; glucose 65.8; xylose 13.6; mannose + arabinose 3.8.

**based on all glucose + one-half (mannose + arabinose)

The results shown in Table 6 meet two of our targets: the ethanol yields are above 350 L/ton and the ethanol concentration is above 6% by volume. However, both the total processing time and the amount of enzyme used exceed our targets. Since we now make our own enzyme from waste newspaper, the amount of enzyme used may be tolerable. Several variations on the above saccharification-fermentation procedure were investigated with the object of meeting all targets.

Variation 1. Five g WOP were added to 100 mL solution containing 43 IU mixed enzymes and incubated at 45°C, and after 24 hours incubation the sugar solution was removed by centrifugation. An additional 43 IU mixed enzymes was added to the residual paper, and after another 24 hours incubation the new sugar solution was separated by centrifugation. The two sugar solutions were combined for analysis. Saccharification by this fed batch enzyme addition was complete, and fermentation of the combined sugar solution was very rapid, being complete is 6 hours to yield 438 L ethanol / ton, the best of any of the procedures, but at very low ethanol concentration, 1.1 % by volume.

Variation 2. In an attempt to raise ethanol concentration, a run similar to Variation 1 was made beginning with 10 g WOP in 100 mL enzyme solution containing 344 IU enzymes, and incubated at 45°C for 24 hours. Then the same amount of enzyme was added 2 more times at 24 hour intervals. Ethanol yield at 24 hours was 411 L/ton, at 4.0 % ethanol by volume.

Variation 3. In this procedure, 20% WOP concentration was obtained by adding the paper and enzymes in 2 equal portions at 6 hour intervals for a total of 1376 IU enzyme. Fermentation was slow, ethanol yields being 336 L/ton at 96 hours, and ethanol concentrtion reaching 6.7 % be volume.

Variation 4. This is low solids, fed batch WOP, 2 g WOP plus 138 IU enzyme being added 10 times over 5 days. After 24 hours fermentation, ethanol yield was 375 L/ton, at 6.8 % ethanol by volume.

None of these variations met all the performance targets. The results shown on Table 6 are the best so far in meeting these targets.

4.3 Bioconversion with Laboratory-Prepared Cellulase

Enzyme solution was prepared using waste newspaper-derived inducer in the 2.5 L fermenter (Figure 00) modified to provide a slow air flow over the fungal fermentation. The solution contained 1480 IU and 1.92 g protein per liter. To 100 mL enzyme preparation (a) after filtration, or (b) without filtration, was added 5 g WOP + 1.95 IU cellobiase. After adjustment of pH to 4.8 and incubation at 45°C for 6 hours, the suspension was cooled to 37°C and 1 g yeast + YMP were added. Anaerobic fermentation yielded ethanol at the rates of (a) 292 and 359 L/ton WOP in 24 and 48 hours; and (b) 319 and 400 L/ton WOP in the same periods. The laboratory-prepared enzyme is thus effective, but still need cellobiase. It is more effective when not filtered, but used as the entire enzyme producion mix, which may save added nutrients.

In an inital run in the 2.5 liter fermenter, commercial enzyme was added fed batch. To 1000 mL enzyme solution containing 2408 IU cellulase and 275 IU cellobiase and the usual additives, was added 100 g WOP, the pH was adjusted to 4.8 with lime and the mixture incubated at 45°C. At 24 and 48 hours, fresh enzyme was added in the same amounts. After a total of 96 hours at 45°C and a total of 8049 IU enzyme, the mix was cooled to 37°C and 20 g yeast + YMP were added. During 6 and 24 hours of fermentation, ethanol was

produced at the rate of 324 and 391 L/ton WOP, and 3.2 and 3.9 % by volume. Samples taken at 72 and 96 hours of fermentation showed no increase of sugar production, in both cases about 71 % of theory, suggesting that fermentation could have begun 24 hours earlier.

Another run was made in which the 2.5 liter fermenter was used for both cellulase production and for fermentation. The enzyme solution so made contained 1480 IU and 1.92 g protein/L. To 1 liter of this enzyme solution was added 91.5 IU cellobiase and 50 g WOP, plus a few drops of Triton X100, pH was adjusted to 4.8 and the suspension was agitated in the reciprocal shaker at 45°C for 6 hours. It was then cooled to 37°C and yeast and YMP were added. After 24 hours fermentation, ethanol yield was 340 L/ton WOP, at 1.7 % ethanol by volume. At this time an additional 50 g WOP was added plus an additional 91.5 IU cellobiase, and agitation was continued for another 24 hours, resulting in an ethanol yield of 320 L/ton WOP at an ethanol concentration of 3.2 % by volume. These yields, while somewhat lower than those obtained in the initial run, are good considering that much less cellulase was used, 1480 IU compared to 7224 IU cellulase, and 183 IU cellobiase compared 825 IU. The usual Vitamin B_{12} and trace elements were not added, and the total time was much shorter, 54 hours compared to 78 or 120 hours. Cellulase useage of 14.8 IU/g waste paper is acceptable.

4,4 Refuse Derived Fuel

The manufacture of RDF on an industrial scale is described in Section 3.2 above, and its analysis and contaminants in Section 3.3. In this section we report the results of bioconversion of RDF to ethanol.

Samples of RDF were supplied by Lundell Manufacturing Company, Inc., Cherokee, Iowa; NEI = National Ecology Inc.,

Timonium, Maryland; and WMI = Waste Management Inc., of Oak Brook, Illinois (sample from BRINI, Sundsvall, Sweden). These were mostly produced in 2 forms, a coarsely divided form, shredded or fluff, or as cylindrical pellets 2 cm diameter by 3 - 10 cm long. Summative analysis was carried out by treating the samples with 72% sulphuric acid at room temperature for 2 hours, the resulting suspension of insolubles in a solution of oligomers being diluted with water with cooling, and then heated to 120°C for 1 hour. After filtering, the sugar solution was brought to pH 5 with calcium carbonate, filtered and analysed by HPLC. From the glucose concentration, the cellulose content of the sample was calculated.

Samples of RDF were shredded, saccharified and fermented. The saccharification medium contained, per liter, 7 g cellulase (Multifect S-850), 3 mL cellobiase (Novozym 188), trace elements, Vitamin B_{12} and 2 mL Triton X100, and the pH was adjusted to 4.8. Saccharification was carried out in 250 mL shake flasks on a rotary shaker at 150 rpm assisted by a few 6 mm stainless steel balls. To 100 mL of this saccharification medium was added 5 g shredded RDF and this was incubated at 45°C for 6 hours. The flask was then cooled to 37°C and 1 g yeast and 5 mL YMP added. Anaerobic closures were attached and fermentation proceeded on the rotary shaker. After 24 hours, a further 5 g RDF plus an addition equal amount of enzymes were added. Samples were taken at 48 and 72 hours and analysed for ethanol (GC) and residual sugars (HPLC).

Table 7. Ethanol from RDF

RDF	cellulose %	ash %	ethanol, L/ton found	theory	% of theory
Lundell pellets	53.5	7.3	316	376	84
Lundell fluff	52.6	3.8	316	370	85
NEI fluff	49.7	10.3	287	350	82
WMI pellets	48.6	13.9	244	340	72
WMI fluff	54.1	9.0	218	380	57

The Lundell and NEI RDF samples gave the best yields, 84 and 82% of theory. The WMI RDF samples are quite diverse, with yields of 72 and 57% of theory. Fermentations were all slow, about 72 hours. The amount of ethanol obtained from these samples varied widely, from a high of 316 L/ton to a low of 218. The 2 Swedish (WMI) samples gave yields fairly close together, 230 ± 13 L/ton. Saccharification and fermentation are apparently inhibited by the contaminants, but the identity of the inhibitors has not been established. The one thing we can be sure of from these results is that considerable variability can be expected, with ethanol yields not obviously related to cellulose content.

The largest waste fractionation plant, the NEI plant in West Palm Beach, Florida, processes 560,000 tons MSW/year. Of this, 75% is recovered in the light fraction, that is 420,000 tons RDF. The bioconversion of this RDF to ethanol would produce 120 million L/year. Such MSW fractionation plants would thus make a significant contribution to fuel ethanol supply.

The amount of enzyme used in these runs, about 20% of the weight of RDF, was chosen so that enzyme availabilty would not be the limiting factor in ethanol production. Ethanol concentrations were about 4% by volume, somewhat below our target of 6% by volume.

4.5 Waste Paperboard Packaging

Table 1, in Section 3.3, reports the analysis of a sample of waste paperboard packaging. Paperboard packaging, as pointed out there, refers to cereal boxes, milk cartons and the like which together form a significant percentage of the paper content of MSW. The analysis (composition) of a "representative" sample of waste paperboard packaging is given in Table 4, in Section 3.3, p 24. It contains 71.3% cellulose and other polysaccharides giving rise upon hydrolysis to 78.4% sugars, with an ethanol potential of 493 liters/ton.

A sample was shredded in an office shredder and liquefied by cellulase enzymes. In shake flasks, 5 g samples of waste paperboard packaging were suspended in 100 mL saccharification medium containing 0.7 g cellulase and 0.3 mL cellobiase at pH 4.8 and 45°C, and incubated for 6 hours. The medium was then cooled to 37°C and 1 g yeast + nutrient YMP were added. Fermentation was carried out anaerobically on a rotary shaker at 150 rpm. Ethanol yield at 24 hours was 321 L/ton of paperboard, 65.1 % of potential, and at 48 hours 347 L/ton, 70.4 % of potential. There is no doubt that the yield can be improved: pretreatment and the use of xylose-fermenting yeasts can each be expeced to raise the yield by 10 %, bringing 48 hour yields to 416 L ethanol/ton, or 84 % of potential. However, even at the lower yields of about 350 L/ton this may well be an excellent route to diverting waste paperboard packaging from landfill to the production of a valuable, marketable product, fuel ethanol.

4.6 Summary of "Best" Process

The questions asked in Section 4.1 Bioconversion Process Background can now be answered, at least in part. Pretreatment before saccharification is not necessary for efficient bioconversion to ethanol of the papers we have studied. Enzymatic saccharification is to be preferred to acid hydrolysis because the yields are much higher and the conditions milder. Cellulase enzymes can be made cheaply on site. The best relationship of time devoted to saccharification and that to fermentation in a modified semi-simultaneous saccharification and fermentation is still a study objective; however, the process proposed in the next paragraph is acceptable. In our practice, saccharification at 45°C combined with fermentation at 37°C has permitted saccharification to proceed at an acceptable rate during fermentation. In a paper presented at the Institute of Gas Technology Symposium on Energy from Biomass and Wastes (1987) E.C. Easley

and colleagues reported fermentation carried out at 40°C combined with stripping of ethanol as produced. This procedure merits further exploration. Fed batch of paper, and perhaps of enzyme, is better than when these are added all at once, in part because paper solids above 10% are difficult to keep in suspension, but are necessary for high ethanol concentration; however after partial saccharification the mix becomes very fluid and more paper can be added. Saccharification is more efficient at low solids, which combined with a fed batch strategy of paper addition can produce high ethanol concentrations.

A "best" process for bioconversion of waste paper based on what we now know can be specified as follows: a saccharification medium containing enzymes plus additives Vitamin B_{12}, trace elements, Triton X100 is prepared. The enzymes are a mixture of cellulase and cellobiase in the ratio of 70:30 (or 80:20), the amount being about 34 IU/g of initial paper addition. The pH is adjusted to 4.8 with lime, and shredded waste paper is added to a solids content of 8 - 10 %. This is incubated with agitation at 45°C for 6 hours. The mixture is then cooled to 37°C and yeast and YMP (or DU) added, the yeast at 10 - 20 g/L, and anaerobic fermentation conditions established. Agitation is continued for 24 hours when a second equal batch of paper and enzymes is added. Incubation and agitation are continued for 24 to 48 hours. Ethanol should be produced at the rate of 350+ L/ton waste paper at a concentration of 6+ % by volume.

Cellobiase is added to saccharification to hydrolyse cellobiose, a product of cellulose hydrolysis and an inhibitor of it. The use of cellobiose-fermenting yeasts should remove the need for cellobiase addition. Some of these yeasts also ferment xylose to ethanol in good yields, an advantage when hardwood-containing papers are being processed.

In these ways the "best" process described here can be improved by any or all of 3 process procedures: (i) by internal yeast supply of cellobiase; (ii) by pretreatment ahead of saccharification, as reported in the next section Section 5; and (iii) by the use of xylose-fermenting yeasts. The first of these is cost-saving, while the other 2 processes improve yields.

5. Pretreatment

5.1 Purpose of Pretreatment

For studies of pretreatment, shredded white office paper (WOP) was used as a model. WOP prepared in our laboratory was shipped to the laboratory of Professor Esteban Chornet, Dept. of Chemical Engineering, Universite de Sherbrooke, who carried out pretreatment in his vapour cracker (see Figure 5), and the resulting pretreated fiber was returned to the Toronto laboratory for enzymatic saccharification and fermentation. This work was written up in a paper submitted to Canadian Journal of Chemical Engineering, a copy of the manuscript being here as Appendix 4.

The process step of pretreatment is necessary in processing such lignocellulosics as wood and agricultural residues. The term is usually applied to a brief exposure, from a few seconds to a few minutes, to steam under pressure at 150 - 240°C. At the end of pretreatment, pressure is released suddenly as a "steam explosion". In pretreatment of wood, hemicelluloses are largely made soluble in water and lignin at least partially in sodium hydroxide solution. This effect is limited to processing hardwoods and agricultural crop residues, where xylose can be completely solubilized and much of the lignin made alkali-soluble. However, with coniferous wood species it is necessary that an acid catalyst be employed in steam explosion pretreatment. Almost any acid will do. Sulphuric acid has most commonly been used: we prefer SO_2. Some recent work has suggested that CO_2 may be so employed. If we can confirm this, using up fermentation CO_2 in pretreatment would add to the benefits of bioconversion of lignocellulosics, including waste paper.

When processing waste paper or MSW or RDF, pretreatment also acts as a sterilizer, reducing the possibility of bacterial contamination.

Steam explosion pretreatment also has the advantage of separating the fibers of wood or other lignocellulosics and making their complete separation easier. The separation of fibers is of some importance, since it represents improved access of the enzyme and the other components of the saccharification medium to the cellulose. It has also been claimed, but not established, that steam explosion pretreatment improves accessability of the cellulose molecules, particularly the crystalline cellulose to saccharification enzymes. This would be expected to improve the rate of saccharification, which is at best a slow process.

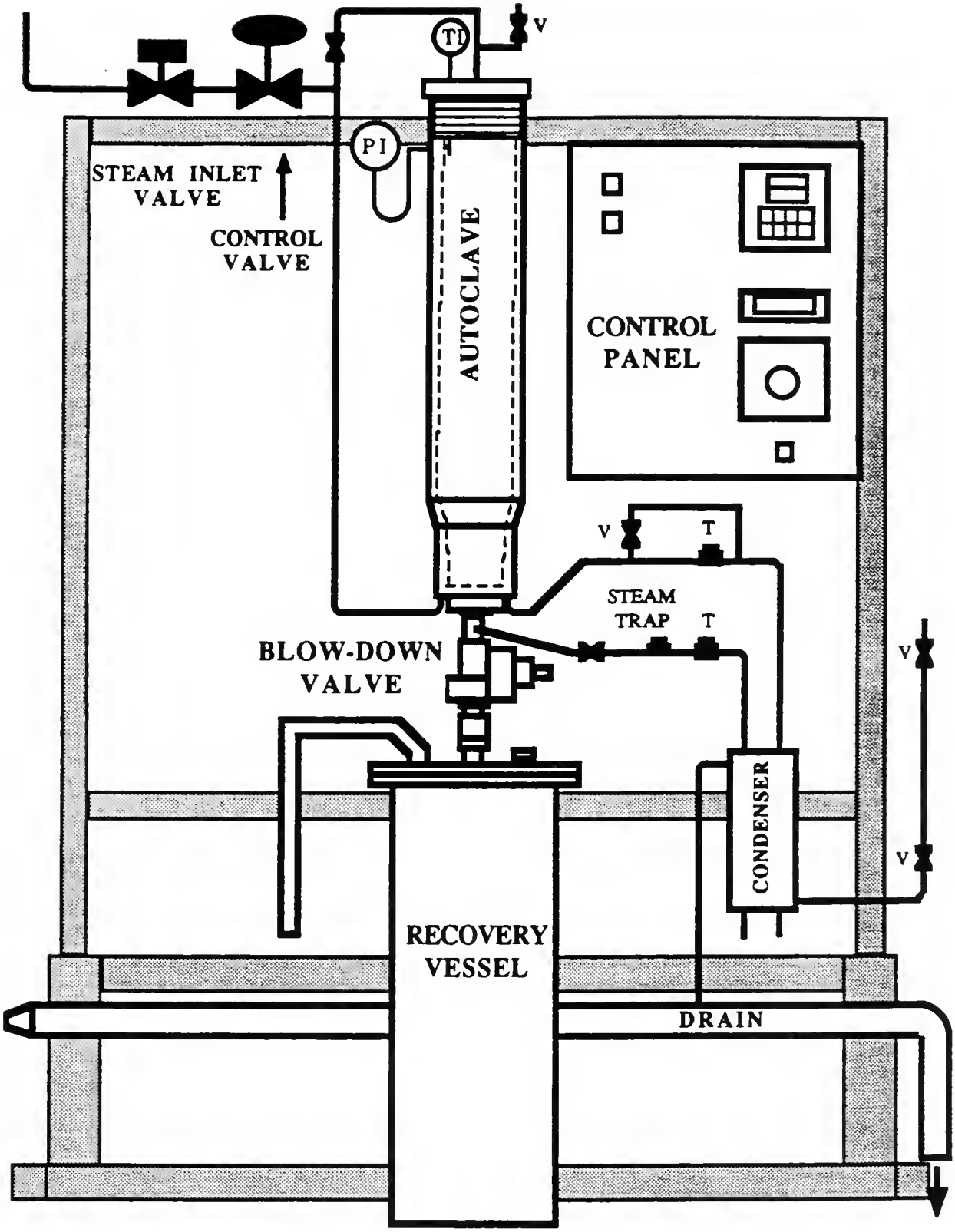


Figure 5. The Vapour-Cracker

We have not considered pretreatment to be important in processing waste paper, since in the pulping process whereby paper stock is made, the wood or other feedstock is subjected to fairly high temperatures, about 160°C, in the presence of chemicals, sulphite acid or sulphate alkali, which should constitute an effective "pretreatment"; and we have shown in Section 4 above that quite good yields of ethanol can be obtained from waste paper without any pretreatment. These yields, in the range of 350 - 400 L/ton waste paper, are only about 70 - 80 % of theoretical yields. Also, rates of saccharification might be improved by pretreatment, hence the experimental runs to be reported here.

5.2 Alternative Pretreatment Equipment

All forms of pretreatment equipment are based on high temperature (150 - 240°C), short time (20 - 200 seconds) processing. There are several main designs:

(i) The earliest design employed for pretreatment is the Masonite gun, widely used for many years in the preparation of fiberboard. In a vertical, heavy-walled pressure vessel of about 0.85 cu m volume, wood chips are heated rapidly by live steam to 800 - 1,000 psi (5.5 - 7.0 MPa, 55 - 68 atmos) and when the desired pressure has been reached, a quick-opening valve releases the contents of the gun to atmospheric pressure. Much destruction of the wood structure is achieved by the sudden release of pressure. The Masonite gun principle has been employed on a very large scale in making rayon grade cellulose from hardwoods by the prehydrolysed kraft process. Professor Chornet's vapour cracker is a variant on the Masonite gun.

(ii) Stake Technology, an Ontario company, has developed a continuous feeding mechanism which enables wood chips or other cellulosics to be fed at rates of about 4 tons/hour to a horizontal pressure vessel where it is held for about 3 minutes at about 2.4 MPa, and then discharged through an intermittent quick-opening valve. The Stake reactor is installed in the IFP pilot plant at Soustons, as pretreatment of lignocellulosics ahead of enzymatic saccharification and fermentation. It is

also being increasingly considered for the preparation of mechanical pulp from wood.

(iii) The Wenger cooker-extruder is a very high pressure, about 7 MPa, steam treatment used primarily for processing foods. It is a continuous processor which accepts wood chips; straw, etc. One of these was installed in the BioHol laboratory in Toronto, and used extensively for pretreatment of cellulosics.

(iv) The St. Lawrence Reactors pilot plant at Kincardine, Ontario, has a continuous plug flow reactor for short time high temperature acid treatment of lignocellulosics. This equipment was previously installed in the BioHol laboratory in Toronto. It has established itself as valuable in the liquefaction of starch; lignocellulosics, however, need to be finely divided to be processed by this equipment.

(v) The Sunds Defibrator, long established world wide in the production of fibreboard, is being used for pretreatment in the pilot plant of TVA, Muscle Shoals, Alabama.

(vi) The Flash Hydrolyser is being used in Europe for pretreatment in the production of ethanol from wastes, as part of the BIOL process of ZellPlan, Munich. It is also a short time-high temperature reactor employing steam or acid catalysed steam. Its main advantage over other such equipment is that the steam is recovered at high pressure to be used elsewhere in the process, for example in distillation.

5.3 Effect of Pretreatment on Bioconversion of Waste Paper to Ethanol

Waste office paper was processed in a mini-vapour cracker located at the Université de Sherbrooke using several different sets of conditions. The resulting fibres were sent to the University of Toronto where they were saccharified by enzymes and the resulting sugars fermented by yeast to ethanol. The waste office paper was shredded to pieces 0.2 - 2 cm width and 2 - 6 cm length. Moisture was 6.8 % by weight. This feedstock was treated in 2 different ways prior to the steam treatment: (i) about 1 kg was soaked in water under vacuum until the cellulosic material was saturated and any occluded air removed; and (ii) another 1 kg was soaked in a solution of 0.5 % sulphuric acid under vacuum. The consistencies used in

the soaking/impregnation routines were 5% by weight of solids. The suspensions were drained and the wet material having typical moisture contents of about 70% was divided into fractions weighing 400 - 500 g. These fractions were kept at 4°C until their introduction into the reactor where the steam treatment is conducted.

The reactor assembly used, named mini-vapour-cracker, is shown in Figure 5. It consists of a 4 L autoclave preheated by steam circulating internally and through its jacket which ensures a uniform temperature. Once the autoclave temperature was stable at the desired level, the waste paper was introduced into the autoclave through the top closure. The autoclave was then sealed and saturated steam introduced at the desired pressure. A thermocouple installed at the center of the bottom third of the autoclave measured and recorded the temperature of the wet material as it rose to the steam temperature. After a prescribed steaming time, the discharge blow valve was suddenly opened by a pneumatically operated actuator and the treated material was ejected from the autoclave into a recovery vessel containing 1 liter of water. The suspension was vacuum filtered and cake and filtrate were stored at 4°C for further processing and analysis. The moisture content of the cake was 62 - 74 %. The yield of fiber was consistently above 90 %. The treated material was well defibered for enzymatic saccharification. In all the runs reported here the fibers were held at the prescribed temperatures for 2 minutes. Temperatures ranged in 10° steps from 170 to 220°C. The treated fibers were packed in ice and despatched to the University of Toronto for saccharification and fermentation.

The saccharification medium contained, per liter: 7 g cellulase, 3 mL cellobiase, trace elements, a small amount of Vitamin B_{12} and 2 mL Triton X100, with the pH adjusted to 4.8. Saccharification was carried out in 250 mL shake flasks containing 100 mL medium on a rotary shaker at 150 rpm, assisted by a few 8 mm stainless steel balls. To 100 mL of this medium was added 5 g of a steam-treated sample (dry weight) and the suspension incubated at 45°C for 6 hours. The flask was then cooled to 37°C and to it was added 1 g yeast and 5 mL YMP. Anaerobic closures were attached and fermentation proceeded on a rotary shaker at 150 rpm. Samples taken at 24 and 48 hours were analysed for ethanol and residual sugars.

The results are shown in Tables 8 and 9. The original WOP contained 88.1 % polysaccharides, of which 69.7 % was glucan, 14.4 % xylan and 4.0 % mannan + arabinan, the remainder being acid insolubles and moisture. The ethanol potential of this paper is calculated to be 504 L/ton.

Table 8. Steam Treatment of Waste Office Paper in the Mini-vapour-cracker for 2 minutes after Presoak in Water under Vacuum

		Ethanol, L/ton	
No.	Temperature °C	24 hour	48 hour
Control, no pretreatment		322	357
1.	170	303	367
2.	180	301	354
3.	190	303	359
Average 1 - 3		302	360
4.	200	327	375
5.	210	324	380
6.	220	324	372
Average 4 - 6		325	376
Average 1 - 6		314	368

Table 9. Steam Treatment of Waste Office Paper in the Mini-vapour-cracker for 2 Minutes after Presoak in 0.5 % Sulphuric Acid Under Vacuum

		Ethanol, L/ton	
No.	Temperature °C	24 hour	48 hour
Control, no pretreatment		322	357
1.	170	391	402
2.	180	352	430
3.	190	358	428
Average 1 - 3		367	420
4.	200	416	460
5.	210	450	468
6.	220	448	453
Average 4 - 6		438	460
Average 1 - 6		405	440

The results in Table 8 show clearly that the water soaking pretreatment in the absence of acid catalyst followed by steam treatment for 2 minutes at these temperatures made very little difference to ethanol yields. At temperatures of 200°C and higher there may be a 10% increase in yields, but considering the complexity of the total process this may not be significant. On the other hand, the results shown in Table 9 show that pretreatment with 0.5% sulphuric acid improved ethanol yields substantially, from 357 L/ton of WOP for the control to 460 L/ton in 48 hour fermentations, an increase of almost 30%. The yields under the best conditions were 91 % of potential. The results also demonstrate that even at temperatures well below 200°C benefits were significant: ethanol yields in 48 hour fermentations were 83 % of theoretical compared to 71 % for the control. Furthermore, the improvement in yields above control was quite noticeable in the 24 hour results: the yields were all better than the 48 hour controls even at the lower temperatures. At the higher temperatures the 24 hour fermentation yields were 95 % of the 48 hour yields, suggesting that the marginal value of the longer fermentation times may not be justified under these conditions.

The filtrates taken following treatment in the mini-vapour-cracker contained no sugars in the samples which had been water soaked, while in those which had been soaked in acid the dissolved sugars, mostly xylose, amounted to 3 - 4 % of the fiber. About 6 % of the fiber remains in solution as residual sugars after fermentation in the water presoaked runs. In the acid presoaked runs 6 % of the fiber remained as residual sugars after fermentation at the lower temperatures, but at the higher temperatures residual sugars decreased to 1.6 % at 220°C. At these processing conditions xylose and galactose were probably dehydrated to furfurals.

While pretreatment with 0.5 % sulphuric acid is shown here to be beneficial, in a previous study gaseous SO_2 was added to a Stake reactor and the resulting fibers were readily saccharified by enzymes. The replacement of sulphuric acid by gaseous SO_2 added directly to the reactor would result in better contacting and a simpler process. In the present work the appropriate equipment for the addition of gaseous SO_2 to the reactor was not available. The same idea may apply to carbon dioxide as pretreatment catalyst, but we have not yet explored this.

To summarize: the vapour-cracker has been shown to be simple and reliable equipment for pretreatment ahead of saccharification of waste paper. When impregnated with 0.5 % sulphuric acid, waste paper steam treated at 170 - 220°C gave yields of ethanol 23 % higher than controls with no pretreatment upon enzymatic saccharification and 48 hour fermentation by yeast. At temperatures above 200°C the ethanol yields approached theoretical potential. At the higher temperatures nearly all benefits were observed in the first 24 hours of fermentation, ethanol yields being 95 % of 48 hour yields, suggesting that the marginal value of the longer fermentation time may not be justified. The suggestion is also made that the addition of gaseous SO_2 to the vapour- cracker in place of sulphuric acid pretreatment would simplify the process.

6. Enzyme Supply

6.1 The Cellulase Complex in Saccharification of Waste Paper

In our process as described in Section 4 above and in Appendices A-3, A-4 and A-5, waste paper or RDF with or without pretreatment (Section 5 above) is saccharified by enzymes to a solution of sugars. Saccharification appears to take place in 2 stages, liquefaction preceding saccharification proper as it does in the starch-to-glucose industry; the conversion of solid paper to a liquid slurry takes place rather quickly, within a few hours, under appropriate conditions, usually pH 4.8 ± 0.4, 45°C ± 5°C with appropriate additives as described. Agitation is provided by rotary or reciprocal shaker-incubators, and about ten 8 cm stainless steel balls are usually added to improve solid-liquid contact. For larger volumes such as 1 L saccharification medium, a useful bioreactor has been described (Figure 5). Under the best conditions, while liquefaction is rapid, saccharification is slow and incomplete. This is due to the inhibitory effect of the newly-formed sugars on enzymatic activity. In order to reduce this inhibition, liquefaction is permitted to proceed at 45°C for 6 ± 4 hours, whereafter the temperature is reduced to 37°C, nutrients, for example diammonium phosphate and urea, and yeast are added. Fermentation, converting the sugars to ethanol, and saccharification proceed simultaneously. The temperature chosen, 37°C, is a compromise, too warm for best growth of the bakers' yeast employed and too cool for best enzymatic activity.

However, it "works." It would undoubtedly be better with a thermophilic yeast, especially a strain which fermented optimally in the temperature range 40 - 45°C. The search for such a yeast continues.

The so-called cellulase enzyme is a complex of at least 5 enzymes, cellobiohydrolase I and II, endoglucanase I and II and β-glucosidase (cellobiase). Of these 5, only the cellobiohydrolases attack solid, insoluble cellulose, reducing it to soluble glucose polymers of different chain lengths, which are attacked by the endoglucanases to form mainly cellobiose. The cellobiose so formed is a product-inhibitor of the other enzymes in the complex. Cellobiose is not fermentable by bakers' yeast. For the hydrolysis reaction to proceed to fermentable glucose, the cellobiose must be hydrolysed, and this is the role of the β-glucosidase. In the presence of a cellulase containing all of these enzymes in the proper proportion, saccharification proceeds to monomeric fermentable sugars, or in the presence of yeast, to ethanol.

The effectiveness of the enzyme also depends on the concentration of the substrate, in this case waste paper, being much more effective at low solids concentrations. For best results, the waste paper is added in batches ("fed batch") to keep solids concentrations low at all times while saccharification and fermentation proceed.

The cellulase complex is made by many fungi and bacteria. The fungus we use, Trichoderma reesei, is one of the best producers of cellulase. Unfortunately, the complex it makes is too low in β-glucosidase and in much of our work we add this enzyme to the saccharification medium (but see Section 6.3 below). The hydrolysis or saccharification of corn or other grains follows much the same route: liquefaction by one form of amylase enzyme and saccharification by a different amylase enzyme. Generally, the first stage is quite brief, about 2 hours, followed by the slower saccharification. So for those in the corn syrup or glucose industry this is a familiar sequence of events. Manufacturers of corn syrup or corn ethanol nearly all buy their enzymes which are low enough in price to have only a minor effect on product price. However, the price of cellulase enzymes is too high to permit purchase of the enzymes for large scale operation: they need to be made on site. As explained in the next section Section 6.2, the aim is to have the same effectiveness per cost unit as the starch enzymes have, a goal made more difficult by the many decades of

industrial experience in the manufacture of starch enzymes. Furthermore, it may not be good enough to make cheap cellulase enzyme unless it has an adequate amount of β-glucosidase.

We see the production of enzymes as the most important problem to be solved for profitable bioconversion of waste paper to ethanol, and much of our research effort is now devoted to it.

How much enzyme is required? In a recent review of cellulase production research, H. Esterbauer and his colleagues surveyed cellulase enzymes from a variety of sources, and concluded that 50 g of enzyme protein were required per kg cellulose. To make this amount of enzyme, their calculations show about 200 g carbon source to be required, or in other words about 10 % of crude enzyme on the weight of waste paper and about 20 % of carbon source for the fungus to consume for each kg of waste paper. Esterbauer's otherwise excellent manuscript suffers from the fact that he did not take into account the importance of β-glucosidase, which can reduce his enzyme requirement to half, that is 100 g crude enzyme/kg waste paper. Our process design is based on the latter number. At a recent conference on ethanol sponsored by the Ontario Ministry of Agriculture and Food, Mr. Pat Foody, speaking for Iogen, predicted that by 1995 their company would be producing cellulase enzymes at a cost comparable to starch enzymes. His enzymes will be needed only at the rate of 2,000 IU/kg cellulose, and this amount can be produced from about 10 g enzyme (1 % on cellulose), or 20 g carbon source (2 % on cellulose, my calculation from his numbers). This is about 5 times better than our design allowance and will, if achieved, represent a substantial contribution to a cellulose ethanol industry. We hope by 1995 to do equally well here.

There are at least 15 companies making and marketing cellulase enzymes, of which 4 are in Japan, 3 in the U.S., and 2 in Finland. For many years we obtained our laboratory supply of cellulase enzymes from Japan, and more recently from Finland. In Canada, Techtrol (Iogen), Ottawa make and sell cellulase enzymes with reported current sales of $3 million per year. Foody expects a large increase in sales over the next few years. The enzyme is produced in the U.S. by processes developed here. It is used in the food industry to clarify beverages with residual fiber. These enzymes are now finding their way into the pulp-paper industry where they are used

to reduce the amount of chlorine needed for bleaching. Thus the manufacture of cellulase has become a burgeoning industry, which should result in lower prices in the coming decades. It remains to be seen, however, whether these expected lower prices will meet our objective of enzyme cost equivalent to that in the corn syrup industry.

6.2 Analysis of Commercial Enzymes

Cellulase activity is usually measured by a method prescribed by the International Union of Pure and Applied Chemistry (IUPAC) in which filter paper is saccharified under specified conditions and glucose production is measured, resulting in a reading of International Units (IU) or Filter Paper Units (FPU), usually in units per mL of the cellulase solution being tested. We have not been satisfied that the IUPAC test corresponds properly to the cellulase activity to be expected with waste paper. We have therefore used, in addition to the IUPAC test, an effectiveness test in which waste office paper (WOP) is used as substrate, and the saccharification conditions employed are those which have become our standard process conditions for saccharification of waste paper. In addition, we determine protein in our cellulase preparations in order to calculate protein specific activity, and where appropriate, β-glucosidase by a different IUPAC method.

To establish a base line for our studies of enzyme production, enzymes have been obtained from a number of sources and these have been assessed for eznyme activity and protein content, with the results shown in Table 6. All of these enzymes have a low protein content, Econase (Alko, Finland) having the highest of the solid enzymes at 55.3 %, while Celluclast SP 122 (Novo, Denmark) has the lowest, 20.8 %. The percentage of protein is an indication of the degree of purification of the enzyme. Too much purification could result in the loss of isoenzymes, which may, perhaps, explain why Econase, with the highest protein content, has the lowest specific activity, that is activity per g of protein, while Celluclast SP 122, with the lowest protein content of the solid enzymes, has the highest specific activity. Four of the solid enzymes have specific activities in the range of 908 - 1200 IU/g protein, while Econase has 544 IU/g protein. The 2 year old sample of Meicelase has not lost any significant activity during storage. Of the liquid samples, Spezyme (Genencor, U.S.) has the highest speciic activity, 1703 IU/g protein, almost 50% more than the next highest.

Table 10. Analysis of Commercial Cellulase Enzymes

		Protein		Lactose*	Sugars + Lactose
	IU/g	%	IU/g	%	g/g enzyme
1. Multifect S850					
Finland	344	37.9	908	5,7	0.08
2. Meicelase (1990)					
Japan	335	30.7	1090	24.6	.01
3. Meicelase (1988)					
Japan	308	27.3	1130	23.2	.01
4. Econase					
Finland	301	55.3	544	-	.037
5. Celluclast SP122					
Denmark	250	20.8	1200	-	.033
6. Spezyme**					
U.S.A.	132	7.8	1703	-	.015
7. Cytolase**					
U.S.A.	87	7.8	1122	-	.015
8. Celluclast CCN 3000**					
Denmark	75	8.7	860	-	.020

* lactose is not fermentable by bakers' yeast
** solutions

Meicelase is loaded with lactose, to about half the weight of the enzyme. This is undoubtedly excess inducer, or perhaps added after cellulase synthesis as a preservative. Lactose is not fermentable by bakers' yeast, so the presence of lactose in the enzyme has no effect on the calculation of ethanol yields. Multifect also has a significant amount of lactose, while none of the other enzymes tested had any lactose, although they have a small amount of galactose, not shown, probably derived from lactose inducer.

From these results, Multifect S850 and Meicelase may be considered the best of the solid enzymes, and Spezyme the best of the liquid enzymes.

6.3 Cellulase Production Research

6.3.1 Waste Newspaper-Derived Inducers

There are several cellulolytic mutants of T. reesei which are reported to be good cellulase producers, among them the one we have been using, the mutant developed at Rutgers University Rut C-30. Cellulase production occurs in 2 phases, the growth phase in which a sufficiently high cell concentration is grown on a growth medium, and the enzyme production phase in which cellulase inducers are added to the medium to begin production of the enzyme. There is a small amount of constitutive cellulase in the fungus, which helps get enzyme production started, but otherwise cellulase is an inducible enzyme. The most widely used inducers are cellulose in some finely divided form such as Solka floc or sulphite pulp, or the milk sugar lactose. It has been estimated that the cost of the enzyme is the largest factor in the cost of the enzyme. Much of our work in this area concerns the relative effectiveness of various inducers for cellulase production. Our aim is to find or develop an inexpensive inducer.

In earlier research, we found that partially hydrolysed aspen wood cellulose in the form of cellodextrin (analogous to maltodextrin in partially hydrolysed starch) is an excellent inducer, and that a combination of aspen cellodextrin and lactose was better than either alone. Based on this observation, we formulated our target: the diversion of 10 % of cellulosic feedstock to cellulase production, and that that amount of inducer would produce sufficient cellulase to saccharify the remaining 90 % of the feedstock.

Cellodextrins were prepared by the 72 % sulphuric acid method from pine wood, newspaper and office paper. In addition, alkali cellulose was made from waste newspaper by treatment with 17.5 % sodium hydroxide, and a partially hydrolysed waste newspaper was made using a limited enzymatic saccharification. Table 11 shows the relative effectiveness of these preparations in inducing cellulase formation by T. Reesei Rut C-30. In addition, Table 11 reports the results of inducing cellulase by lactose and by mannose. When using newspaper cellodextrin or partially saccharified newspaper, protein production as an indicator of enzyme production was 2 g/L, while in our effectiveness test these inducers resulted in 66 and 72 % saccharification efficiency measured by sugar formation from office paper.

Table 11. Cellulase Production using Various Inducers

Inducer	Protein g/L	Residual Sugars, g/L	Saccharification Effectiveness, g/L Glucose	Cellobiose	Xylose + Galactose	Mannose + Arabinose	Total %
Lactose	0.66	0.00	4.29	2.12	3.30	0.00	14.49
Mannose	0.24	0.00	1.99	0.78	1.57	0.38	7.04
Newspaper Cellodextrin	2.19	0.30	22.54	11.91	9.44	0.72	66.57
Newspaper Hydrolysate	2.03	0.00	24.53	14.23	8.46	1.53	72.75
Pine Prehydrolysate	0.80	0.49	15.75	8.73	7.53	0.36	48.31
SO_2-pretreated Pine	0.43	0.45	12.11	8.68	5.39	0.06	39.16
NaOH-treated Newspaper	0.32	0.00	7.91	5.83	4.60	0.00	27.30

Since lactose is the inducer most widely used commercially, a series of runs was made producing cellulase induced by lactose in combination with these newspaper-based inducers. Except for the alkali cellulose, the others induced cellulase more effectively. A series of runs was then made with mixtures of inducers containing newspaper cellodextrin. Both mannose and lactose improved cellulase yields compared to cellodextrin alone, giving protein yields of 2.5 g/L and 2.3 g/L, and saccharification effectiveness of 71 and 65 %.

An additive which proved useful in enzyme induction was the sugar sorbose, which for maximum effectiveness needed to be added after 3 days of enzyme synthesis. Under these conditions, sorbose added to partially hydrolysed newspaper raised protein production to 2.64 g/L, and saccharification effectiveness to 93.5 %.

Having shown that waste newspaper properly prepared was a good source of enzyme inducer, the next step was to quantify this finding. Partially saccharified newspaper (psn) was obained by enzymatic hydrolysis of shredded newspaper using 2.3 % cellulase protein at 6 % solids, 50°C, pH 4.8 on a rotary shaker at 150 rpm for 48 hours. At the same time, T. reesei was grown on potato dextrose agar slants at 30°C for 3 days, when sporulation occurred. Inocula were prepared by transferring the conidia to Mandels and Reese medium containing 10 g/L glucose and 1 g/L peptone.

The fungus was grown in flasks for 48 hours at 34°C on a rotary shaker at 200 rpm. A 10 % inoculum was used to initiate cellulase production on the same medium except that instead of glucose psn was used at 10 - 15 g/L. Cellulase was produced at 28°C, initial pH 5.0. At the end of 6 days, the cellulase solution was filtered, analysed and tested for saccharification effectiveness. About 38 % of psn consisted of water soluble sugars, the rest being mainly cellulose and hemicellulose. Among the water soluble sugars, glucose was 27 %, glucose dimers 2.7 %, trimers and higher 0.87 %, xylose 3.5 %, mannose + arabinose 3.6 % and galactose 0.3 %, leaving water-insoluble carbohydrates at 62 %. Production of protein by psn was 2.0 g/L. When sorbose was added after 3 days, protein production was 2.5 g/L. These results are shown on Figures 6 and 7. It was also shown that the amount of sorbose used in this set of runs, 5 g/L, was far too much, and that 1 g/L would have been enough.

The rate of formation of enzyme is shown on Figure 8. The FPU of the filtrate increased at a more or less constant rate during the first 6 days, and increased further at a slower rate during the next 4 days, unlike the protein profile which increased rapidly during the first 4 days and then levelled off. Thus the enzyme protein produced in the first 4 days had lower specific activity than the enzyme produced later. Figure 8 also shows some xylanase activity, as well as β-glucosidase activity as reflected in glucose:cellobiose ratios. The latter increased rapidly from Day 3 to Day 6, and levelled off about Day 9. Saccharification effectiveness as shown by total sugar production reached about 60 % of the maximum value within the first 2 days, increased steadily in the next 5 days and then levelled off.

Figure 9 compares the saccharification effectiveness of this cellulase preparation with that of commercial enzymes, at the same amount of protein. The enzyme prepared from psn is at least as effective as the best commercial enzymes. The addition of a small amount of β-glucosidase improved effectiveness by almost 20 %.

In our runs, cellulase production was usually terminated by the end of the sixth day. However, it was observed that the inducer was not used up by that time. Therefore cells and lignocellulosic residue were filtered and incubated for another 6 days. More cellulase, an additional 1.2 g/L protein, was produced. These results showed that it was possible to prolong the period of enzyme secretion with a limited amount of inducer.

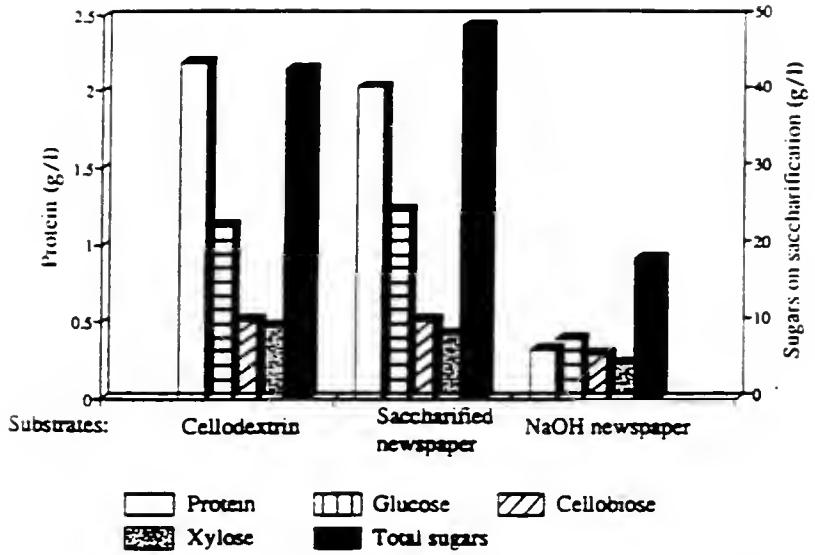


Fig. 6. Cellulase production induced by newspaper-derived substrates (15 g/litre) and saccharification performance of the cellulases.

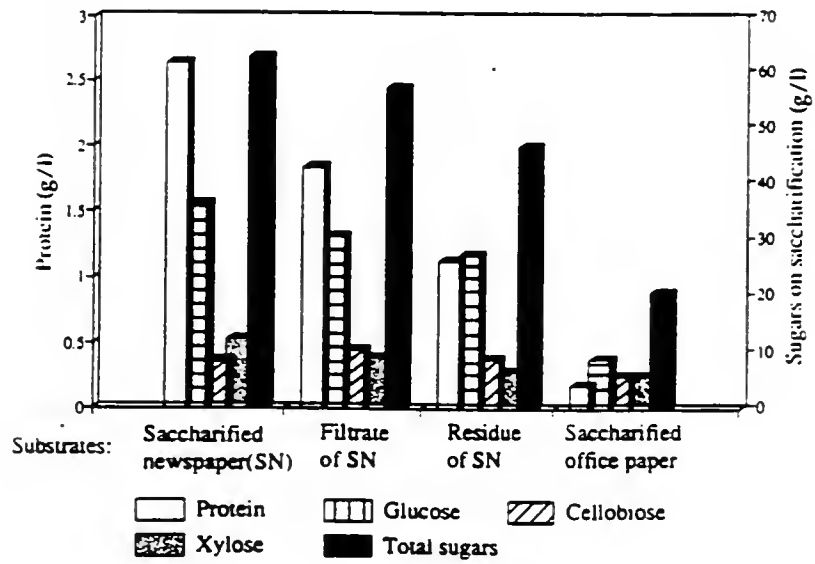


Fig. 7. Cellulase production from newspaper derived substrates (10 g/litre) with addition of sorbose (5 g/litre) at 96 h. and saccharification performance of the cellulases.

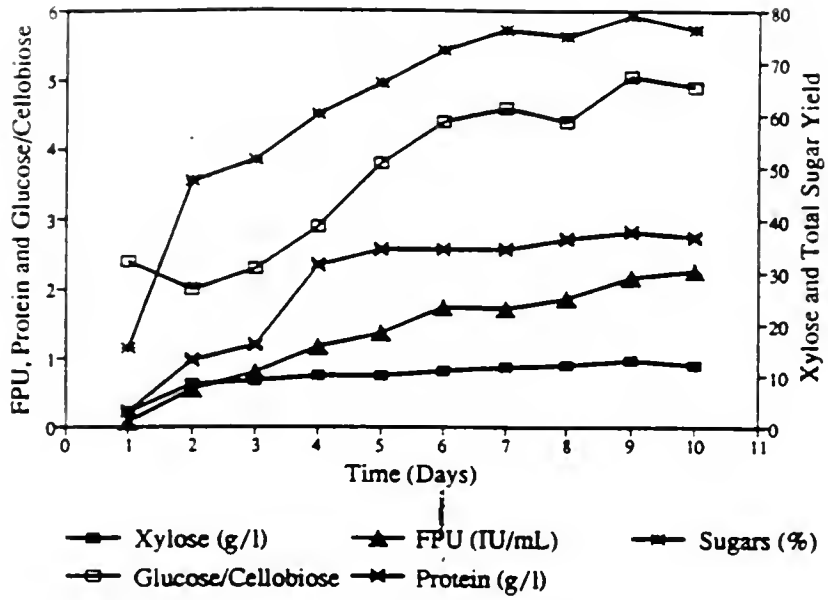


Fig.8 Kinetics of Cellulase Formation

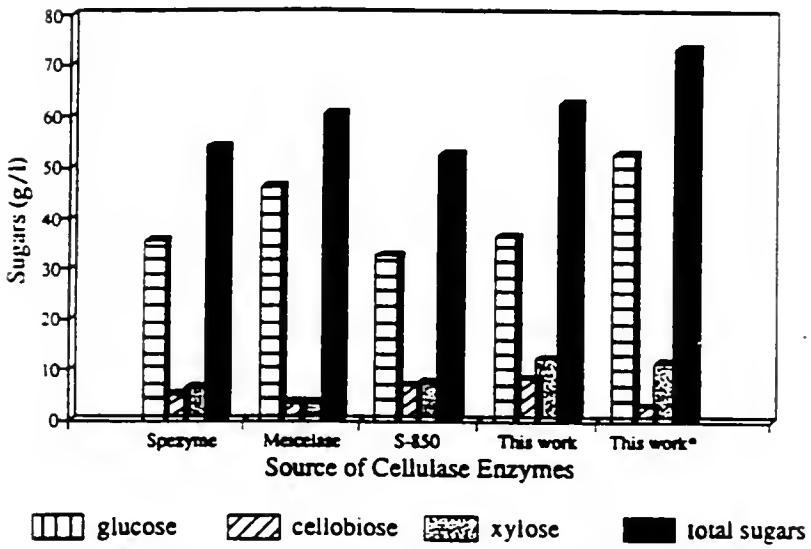


Fig. 9. Comparison in sugar yields from saccharification of 9·34% office paper by different cellulase enzymes. * With addition of 0·01 ml of β-glucosidase for each gramme of paper (dry).

From our studies we can conclude that psn is an effective enzyme inducer: from 1 g psn as inducer + 0.07 g sorbose, the cellulase produced was effective in saccharifying 8 g waste paper yielding 5.4 g fermentable sugars, or 6.3 g fermentable sugars when supplemented with a small amount of β-glucosidase. Our target called for 1 g inducer to produce enough enzyme to saccharify 9 g waste paper. This was easily achieved by recycling cells and unconsumed inducer. Cellulase produced by such recycle using the same total of 1 g newspaper + 0.07 g sorbose saccharified 16 g waste paper, producing 10 g fermentable sugars, or more than 11 g with β-glucosidase supplementation. This suggests that the amount of waste paper feedstock to be diverted to cellulase production may be much less than our targeted 10 %.

The work just outlined is presented in more detail in Appendix A-6 "Cellulase Production Induced by Carbon Sources Derived from Waste Newspaper."

6.3.2 Starch-Derived Inducers

When waste newspaper is used as an inducer for production of cellulase by T. reesei, it is only partially consumed. The enzyme solution is recovered by centrifugation or filtration, a step which results in some loss of enzyme. To avoid this step, a soluble, inexpensive inducer was sought. It was found, after considerable research, that starch could be treated to make an effective soluble inducer. When 1 g starch was partially acid hydrolysed and used for cellulase production, the enzyme so produced, supplemented with a small amount of β-glucosidase, saccharified 15 g waste office paper with high yields of fermentable sugars, in a single cycle, that is no recycle as employed when psn enzyme saccharified 16 g waste office paper, well exceeding our target of 10 g paper per g inducer..

At first glance, starch would seem to be an unpromising source of cellulase inducer, since starch polymer has an α-glucoside configuration while cellulose is a β-glucoside polymer. Our success with starch leads us to believe that the true inducer is a reversion product of glucose resulting from the method of partial hydrolysis. To be used as an inducer, starch must be partially hydrolysed. Enzymatic hydrolysis was less effective in preparing inducers from starch, partial acid hydrolysis being better. The

starch at 30 % solids was hydrolysed by 0.3N HCl at 121°C for 1 hour. Upon using this acid-hydrolysed starch (AS) as inducer for cellulase production, after 6 days under conditions described in 6.3.1 above, protein concentration was 3.7 g/L and saccharification effectiveness was 77 % of theory. Enzyme production was carried out in 250 mL flasks containing 100 mL medium. To go to a larger scale, we adopted 2.5 L fermenters containing 1,000 mL medium (see Figure 4, p 29 and Appendix A-3). After 6 days of cellulase production, waste paper was added to the total mixture. With waste paper derived inducer, protein was 1.8 g/L, and cellulase so produced when used in the bioconversion of waste office paper resulted in 359 to 399 liters ethanol/ton paper in 48 hour fermentations (292 and 319 liters/ton in 24 hours). When partially hydrolysed starch was used as a cellulase inducer, protein content was 1.92 g/L (1,480 FPU/L) and ethanol production from waste office paper was 329 to 392 L/ton in 48 hour fermentations (268 to 334 L/ton in 24 hours. These can be considered satisfactory results at this stage.

The details of starch-based inducer research are reported in Appendix A-7 "Novel Inducers Derived from Starch for Cellulase Production by Trichoderma reesei".

During the course of this research, we found that the addition of wheat bran to the cellulase production medium results in a more effective enzyme. In Figure 10, the best result was obtained when about 1/3 of the inducer prepared from starch was substituted by wheat bran. Whereas cellulase induced by starch derivatives saccharified 15 times the amount of starch used, cellulase produced with this bran substitution saccharified 19 times the weight of the starch used, an increase of more than 25 %. This result led to the thought that whole wheat as a source of both starch and bran may be an effective cellulase inducer under the right conditions, and it proved to be so.

Whole wheat flour was prepared for us by Grain Process Enterprises, Scarborough, taking care to include all of the bran along with the grain. Flours were prepared from soft wheat (12.4 % protein, 71 % starch), and from hard wheat (14.8 % protein, 71 % starch). Wheat bran, for comparison, has 18.2 % protein and 19.1 % starch, + 57.6 % non-starch carbohydrate. These flours were partially hydrolysed with acid in the same

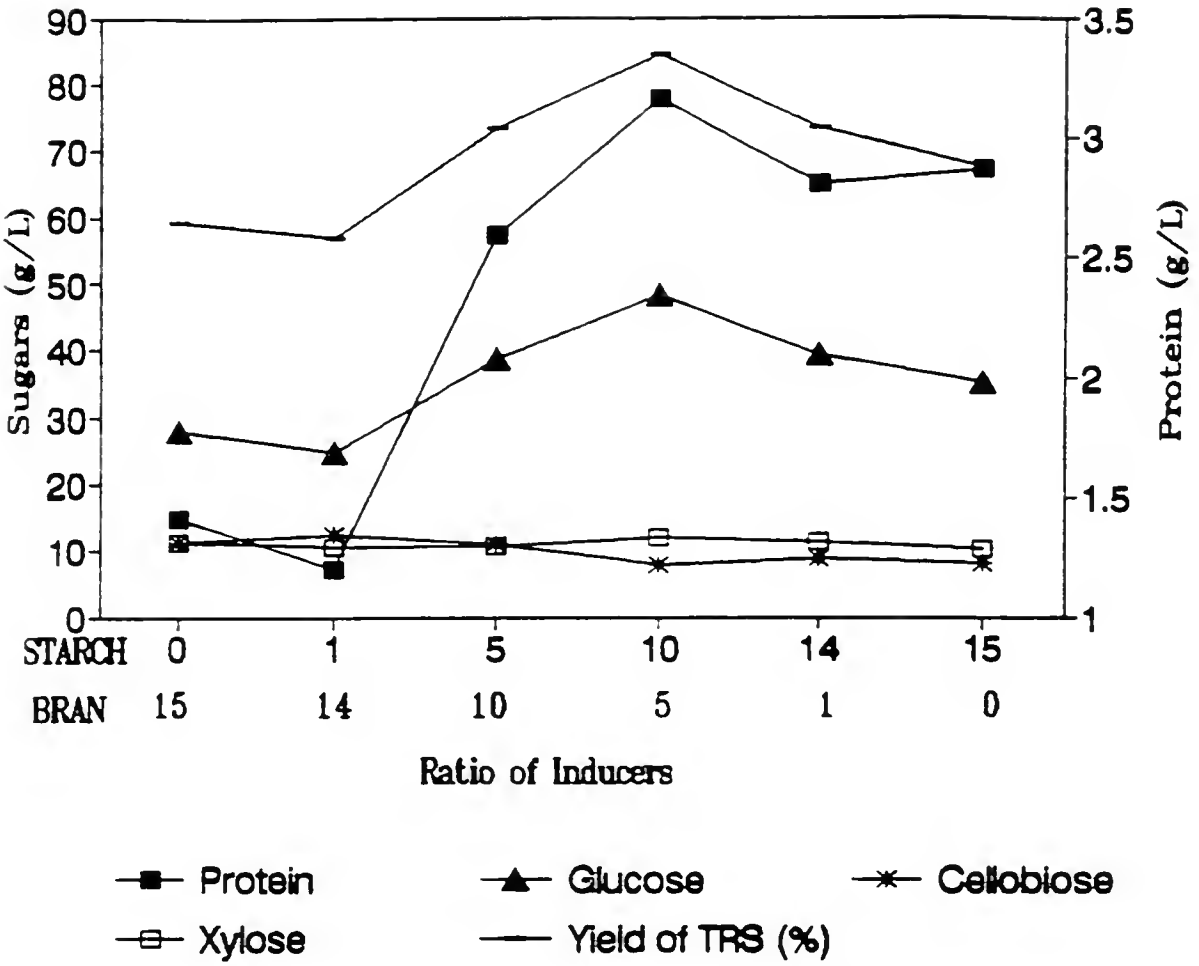


Figure 10. Cellulase production induced by acid-treated starch and wheat bran

When 1 g starch and 0.42 g wheat bran were used, cellulase so produced + 0.1% beta-glucosidase saccharified 19.25 g waste office paper in 9.43 % suspension, yielding 13.88 g TRS or 9.88 g glucose.

was as the starch derivatives described above, and used as inducers of cellulase production by T. reesei. The cellulase so produced, when supplemented with a small amount of β-glucosidase, saccharified waste office paper 13 times the weight of the flour employed. If allowance is made for the percentage of starch in the flour, the results are comparable. Whole wheat flour has a number of advantages in that it is less expensive than starch. It was also found that wheat flour contains buffers, probably protein or amino acids, which provided complete pH control during enzyme production. Furthermore, whole wheat flour provided all necessary nutrient for both enzyme production and saccharification-fermentation. Table 11 compares the effectiveness of the 4 novel inducers we report on above:

Table 11. Comparison of Novel Cellulase Inducers

cellulase inducer	g waste paper saccharified per g inducer source
waste newspaper	16
starch	15
starch + wheat bran	19
whole wheat flour	13

Each of these inducers has its advantages and disadvantages, but each is less expensive than inducers currently employed in industrial cellulase production.

6.4 β-Glucosidase (Cellobiase) Supply.

Cellulase saccharification of cellulose produces a mixture of fermentable sugars and cellobiose, the latter being a dimer of glucose not fermentable by common bakers' yeast. To hydrolyse the cellobiose to fermentable sugar requires another enzyme, β-glucosidase or cellobiase. We wish to avoid the cost of purchased β-glucosidase. Cellobiase is present in our cellulase, as evidenced by production of glucose during saccharification. However, when cellobiase is added to our cellulase saccharification effectiveness is improved. This demonstrates that our enzyme has insufficient cellobiase. The supply of sufficient cellobiase is a matter of some concern in this research.

There are several reports in the literature of enzyme mixtures produced by T. reesei strains which have sufficient cellobiase. We have not been able so far to verify these findings: our home-made cellulase as well as our purchased commercial cellulases are poor in cellobiase. Perhaps we have not yet found the way to optimize cellobiase production by T. reesei In Figure 12 β-glucosidase production peaked rather sharply during cellulase synthesis, and we may have missed the peak. It is therefore important for us to find a process which will address this problem. We are studying 2 different approaches. One is to find a fungal strain which is a rich producer of cellobiase. In the other approach, the problem may be by-passed by employing for fermentation a yeast known to make cellobiase and to ferment cellobiose. We have described such yeasts in an earlier paper (Applied Biochemistry and Biotechnology 18, 325 - 338, 1988). Among these yeasts, Candida lusitania, Pichia stipitis and Brettanomyces clausenii are good candidates the latter 2 because in addition to fermenting cellobiose they also ferment xylose. These deserve further study and we are now assembling cultures of these and other such yeasts, and will use them for fermenting saccharified waste paper without added cellobiase to test their ability to ferment cellobiose.

Our research on cellobiase supply has proceeded rather slowly. However, as of July 1, Shu Chen will begin post-doctoral studies on enzyme supply, both better processes for cellulase production and processes for β-glucosidase. We expect rapid progress.

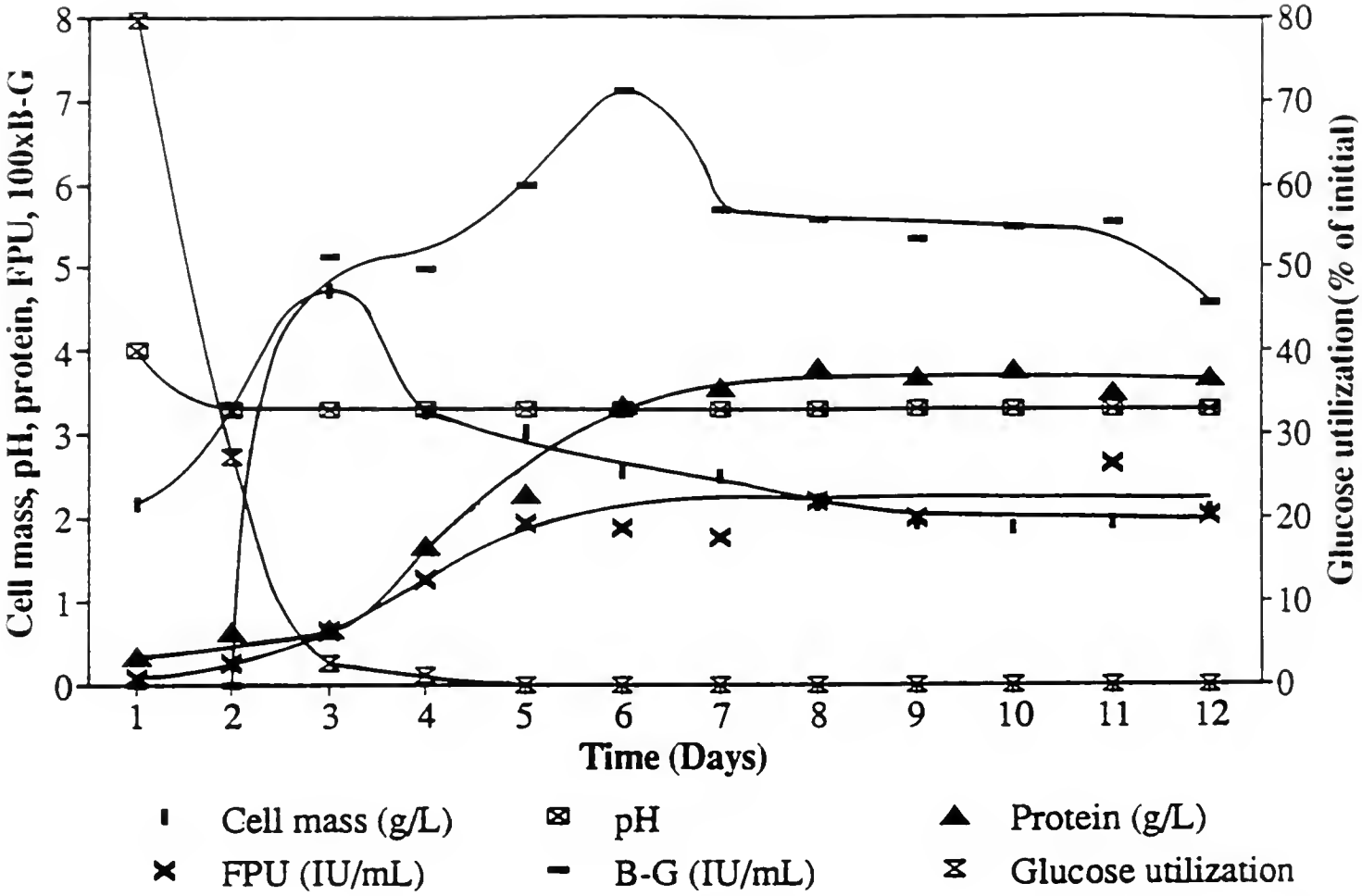


Figure 12 Kinetic behavior of cellulase production on starch hydrolysate (AS3). B-G: beta-glucosidase activity.

7. Environmental Impact

7.1 Landfill Volume Reduction

The waste fractionation systems described in Section 3.2 above claim to divert 80 - 90 % of MSW to valuable products leaving a residue of between 10 and 20 % for landfill (or other disposal). While these systems are expensive, careful consideration may show that they represent the most economical as well as the most effective means for achieving the targets for reduction of the volume of MSW going to landfill set by the Ontario Ministry of the Environment. As far as we are aware, this is not being given serious thought by any provincial or municipal body, even though Lundell, for example, is located in Ontario. This is an oversight which calls for careful study and remedy.

The segregation of ICI paper and the planned segregation of waste paperboard packaging and their conversion to ethanol will also reduce the flow of MSW to landfill. A properly mounted program designed to trap all of Ontario's waste paper for bioconversion to ethanol would also have an impact on the volume of landfill. We estimate that such a program would reduce the volume of MSW to landfill by 40 - 50 % and provide valuable income from the sale of ethanol.

We have thus 2 numbers: 80 - 90 % reduction if an MSW fractionation system, a Materials Recovery Facility, is in place, and 40 - 50 % if office papers, household papers and waste paperboard packaging are segregated and collected in a program modelled after the Blue Box program for old newspapers. Either is of course very welcome; what is needed are careful engineering studies of the costs involved, and of options to the Blue Box program which has proven to be far more expensive than anyone expected. These are problems which can and should be solved, in order to meet the targets of the Ontario Ministry of the Environment.

7.2 Air Pollution and Carbon Dioxide

Experience over many years in São Paulo has proven that the use of gasohol, in that case 20 % ethanol in gasoline, has a remarkable effect in reducing smog and air pollution generally. This heavily industrialized city was a sink of bad air, and it now celebrates clear, blue skies. Such experiences have led some states such as Colorado to mandate the use of ethanol in gasoline during periods of smog.

The paper by Alan Goodall of Statistics Canada, reproduced here as Appendix A-2, has reviewed automobile emissions, and has drawn attention particularly to the resulting high levels of ozone at ground level, and the ozone effect on crops and trees, and, presumably, on human health. He has given estimates of crop losses at several million dollars. He has reported high levels of ground level ozone particularly across southern Ontario, where most Ontarians live. This paper should be required reading for all Ontario policy-makers concerned with the environment, air pollution and transportation. It is our expectation that the displacement of petroleum based gasoline by the environmentally friendly, high octane motor fuel ethanol will have a salutary effect on ground level ozone concentrations, with benefits to crops and to human health.

Fermentation for ethanol production and the burning of ethanol motor fuel each result in the release of carbon dioxide. The cellulose of which paper is made is the result of the fixation and removal of carbon dioxide from the atmosphere by photosynthesis. These 2 reactions, the removal of carbon dioxide from the atmosphere by photosynthesis and its return to the atmosphere as a result of fermentation and combustion exactly balance each other: the amount of carbon dioxide made by the production and combustion of ethanol is exactly the same as the amount of carbon dioxide removed from the atmosphere in the manufacture by the tree of the cellulose of which the paper is made. Hence there is no net increase in atmospheric carbon dioxide as a result of bioconversion of waste paper to ethanol.

In a large plant such as the 40 million liter plant discussed in the section of economic analysis Section 9, carbon dioxide may be collected as a useful, saleable product. Actually, the 40 million liter plant is on the border between profitable and unprofitable carbon dioxide recovery and we have not included its recovery in our plant design, but any larger plant would certainly recover carbon dioxide for industrial, agricultural and food uses. In addition to its use in beverage gasification, carbon dioxide is useful in improving productivity of greenhouses, by increasing photosynthesis. We also foresee the possibility of using carbon dioxide as catalyst in pretreatment within the bioconversion process. From the point of view of bioconversion plant design, this would be a welcome outcome. Use of carbon dioxide in these various ways would bring about a net decrease in atmospheric carbon dioxide.

7.3 Stillage

Every fermentation plant faces the problem of use and disposal of stillage, the still bottoms of the first, stripping column in the distillery. A plant for bioconversion of waste paper to ethanol may have a larger problem than a corn- or beet-sugar based ethanol plant because of insoluble matter such as lignin, ink, etc. present in fermentation, which will find its way to the bottom of the stripping column. A considerable proportion of the stillage, and we have allowed 50 % in our design, may be recycled as dilution to fermentation after screening or filtration. Alternative treatments of stillage for cattle feed have been described in our recent book *Biotechnology of Biomass Conversion: fuels and chemicals from renewable resources.* Stillage may be dried to make Dried Grains and Solubles, or a 65 % concentrate with about 20 % protein which can be sold for cattle fodder. Stillage is also used as a fertilizer and irrigant, where it serves among other benefits to replace depleted minerals in the soil, and to enhance water retention. The recycle of stillage to the soil, however, has limits, because it contributes to soil acidity. In planning industrial bioconversion of waste paper to ethanol, we visualize recycling half the stillage as dilution to the fermentation, and evaporating the remaining stillage to 50 % dryness and selling it as cattle feed. Any unused residue from stillage or other sources could be burned in a Flash Pyrolyser, which would not only safely dispose of a residue but would produce heat useful in distillation or elsewhere in the process.

8. Process Flow Chart and Mass Balance

8.1 Process Flow Chart

The Process Flow Chart given on the next 4 pages is designed for a demonstration ethanol from waste paper plant to be built in Basel, Switzerland. The plant will accept 4 tons per 8 hour day of a 50:50 mixture of waste paper and straw, and produce 400,000 liters of ethanol annually. The Flow Chart given here represents our best concept at this time. There are of course many variations possible, particularly in the areas of pretreatment, enzyme production, heat conservation in fermentation and distillation, and stillage disposal. The concepts for pretreatment and residue

BASEL ETHANOL FROM WASTES PILOT PLANT

OBJECTIVES: The Objectives of this pilot plant are to obtain the necessary data to guide the design and construction of a large scale ethanol from wastes industrial operation, and to define the conditions for fermentation of the pentose sugars in straw.

TECHNICAL DESCRIPTION

PRINCIPAL STATISTICS:

Feedstock: waste paper or a 50:50 mixture of waste paper and straw.

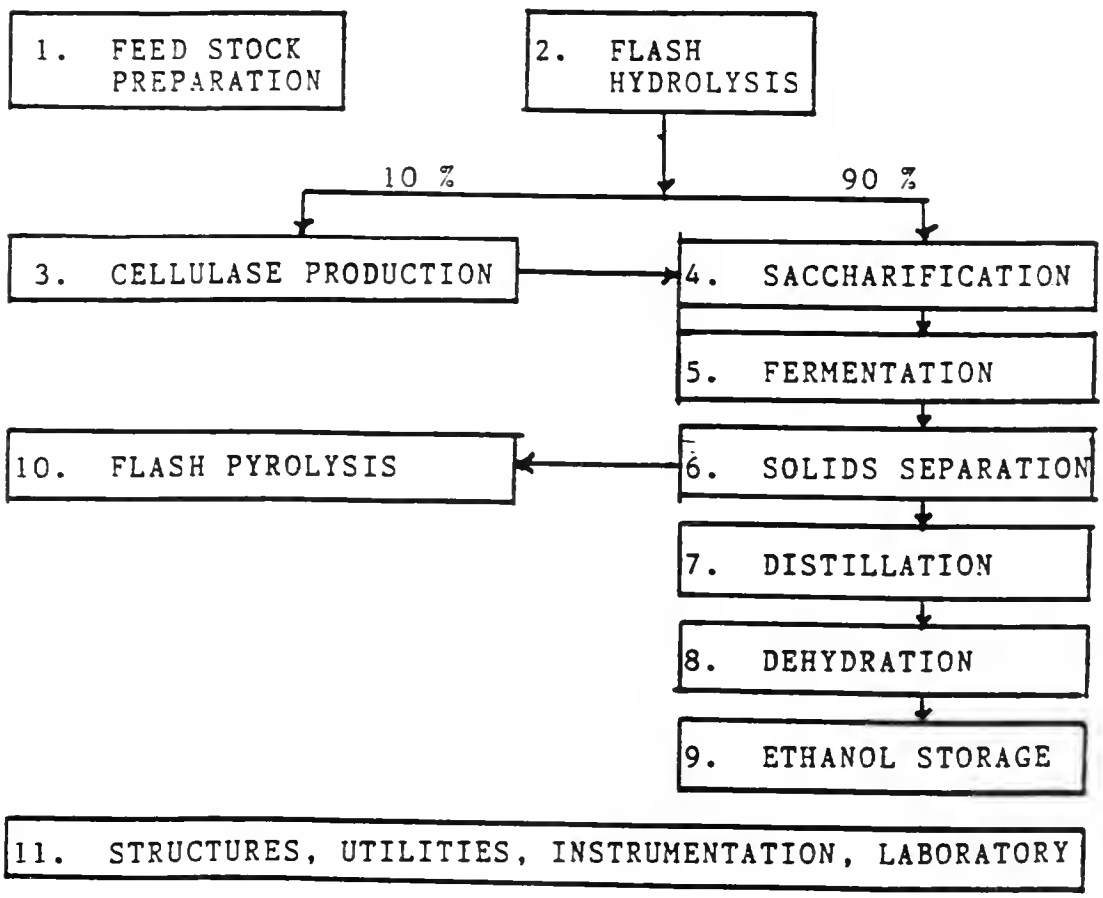
Rate of Feed: 500 kg/h, 8 h/day, 5 dsys/week = 20 tons/week, 1000 tons/a

Ethanol Production: 400 Litres/ton of waste paper, 8 cu m/week, 400 cu m/a

Products: 95% ethanol, or 100% dehydrated ethanol for fuel or other markets

Process: Flash Hydrolysis (FH) followed by Simultaneous enzymatic Saccharification and Fermentation (SSF), on site enzyme production, and effluent treatment by Flash Pyrolysis.

BLOCK FLOW SHEET



Blocks 1 and 2: Feedstock is either waste paper or a mixture of 50% waste paper and 50% straw. If a substantial amount of straw is used, this will affect the fermentation (see Block 5).

Blocks 1 and 2 are provided by ZellPlan, their FH product being the feed for the remainder of the ethanol plant. The final unit in Block 2 is a Live Bottom Bin, containing about 1 day's FH product, that is about 4,000 kg dry weight (less a small loss), at 20 - 50% moisture, in the form of porous granules, bulk density 400 kg/ton (1 ton = 1000 kg). The product of Block 2, the FH fibre from the Live Bottom Bin, is divided into 2 streams, 10 - 12% to Block 3 for Cellulase Production, and 88 - 90% to Block 4 Saccharification. The FH as produced has a pH of 2.5 to 3. This needs to be neutralized with lime for the next two stages, pH 5.5 for the Cellulase Production stream and 4.8 for the Saccharification stream.

Block 3: Cellulase is produced by a green fungus Trichoderma reesei. First of all, the fungus must be grown to a useful quantity, beginning with a laboratory culture worked up in 3 stages of aerobic growth at 34°C to a quantity which will amount to 1.5 - 5.0% of the volume of the medium in the Cellulase Production vessel. The growth phase takes place in a specified medium, and produces very little cellulase. The enzyme is produced when the fungus is induced to make it, which requires an inducer. In our plant, FH product is the inducer.

Cellulase Production is carried out in 2 parallel 30 cu m agitated aerobic fermenters, at 28°C, and requires 5 - 8 days. Medium is added to a fermenter to total volume of 20 cu m, and FH fiber is added steadily at the rate of 500 kg/day until 2500 kg have been added. Temperature, agitation and aeration are continued for 2 more days. At that time, the amount of cellulase produced should be sufficient to liquefy and saccharify the rest of the FH fibre produced that week, that is 17,500 kg.

Block 4: Liquefaction and partial saccharification take place in a 30 cu m vessel with agitation, but no aeration. To this vessel is added one-fifth of the contents of the Cellulase Production fermenter warmed to 45°C, and additional medium at this temperature is added to a total volume of 14 cu m. The acidity is adjusted to pH 4.8, and a small amount of Triton X100 or equivalent surface active agent is added. Liquefaction and partial saccharification proceeds under these conditions for 6 hours, during which 3500 kg of FH fiber is added at the rate of about 800 kg/hour. This generates a sugar solution for fermentation under conditions which allow saccharification to continue to completion.

Block 5: Fermentation takes place anaerobically in a 3-vessel cascade continuous fermentation system maintained at pH 4.8, at 37°C, the system having a total volume sufficient to ferment 2 days' production of partially saccharified FH fiber, a total volume of 28 cu m. The three fermenters are approximately the same size, with the third fermenter somewhat larger than the others such as liquor volumes of 8, 8 and 12 cu m, plus the usual allowance for head space. Ordinary yeast is added to the first fermenter and works its way through the system. The beer issuing from the third fermenter will have at least 3% ethanol, and may under the best conditions have 6+% ethanol, depending upon the ability of the system to maintain the high solids necessary for high ethanol concentration.

If the FH feedstock is made of white office paper, only conventional bakers' or brewers' yeast needs to be employed. Among the trial runs to be made with this SSF system will be one in which a cellobiose-fermenting yeast Dekkaria anomala is added to the second fermenter. If the FH feedstock contains a substantial proportion of straw, a xylose-fermenting yeast such as our Candida shehatae strain needs to be added to the third fermenter. The fermentation of straw-derived sugars requires the presence of a small amount of air. For that purpose, the third fermenter is provided with means to add a controlled volume of air, about 1 cu m/minute.

The operation will include a small yeast production facility in which the nutrient sugars will be supplied by the active fermenters. This serves to adapt the yeast to this sugar medium.

Block 6: At the end of fermentation the beer will contain residual solids derived from lignin, fungal mycelia, yeast and fibers not saccharified. Lignin will be a small proportion, perhaps 2%, of a white paper feedstock, but 10 - 12% of a mixed paper-straw feedstock. All of these solids will now be concentrated in a screw press. This involves a small loss of ethanol, but provides two products: a clean beer for distillation, and concentrated solids for Flash Pyrolysis. The former will enter the beer well which feeds the beer still of the distillation system. The solids will be about 33% dry, and a source of combustible fibers will be added to raise the dry content to 50%. This will be fed to Flash Pyrolysis Block 10.

Block 7: Distillation. The alcohol content of the beer will be stripped by heat in the beer still, which is the first tower of a conventional 3-tower distillation train. The maximum heat recovery will be practiced around the beer still. As much of the still bottoms, the "stillage," will be recycled to earlier stages of the process thereby conserving heat. The two rectification towers serve to concentrate the ethanol to 95%. Or, if an oversize dehydration system (Block 8) is installed, there is considerable energy saving in rectifying the ethanol only to about 90% and allowing the dehydration towers to remove the remaining water.

A fusel oil side stream will be collected, which may be used as fuel, or sold, or may be added to the ethanol.

Block 8: Dehydration. For motor fuel which is to be used directly and not mixed with gasoline, 90 - 95% ethanol (10 - 5% water) is satisfactory. In this case the dehydration facility is not used. However, for blending with gasoline the remaining water must be removed from the ethanol. This is accomplished in a dehydration facility consisting of two parallel towers containing a drying agent, usually a zeolite molecular sieve or cracked corn. The ethanol vapour stream is dried in one column until the effluent from that column shows water, whereupon the ethanol stream is diverted to the other column, and heat is used to regenerate the drier in the first column. The cycle is repeated drying all of the ethanol.

Block 9: Ethanol, either as 90%, 95% or 100%, is stored in accordance with government regulations. Depending upon its market, it may be denatured. Ethanol production is expected to be 1400 L/day, or 7 cu m/week.

Block 10: The solid product of the screw press mentioned in Block 6 above is fortified to about 50% dryness or more, and burned in a Flash Pyrolysis unit. Other means of environmental control include maximum stillage re-use, and it is expected that the small amount of residual effluent will be no problem.

Block 11: All necessary structures, storage areas and tanks, utilities and instrumentation will be provided. A small laboratory will serve to analyse feedstock and product, and provide process control. A small microbiological laboratory will provide and maintain the necessary fungal and yeast cultures.

disposal are based on the equipment invented and developed by ZellPlan, an engineering company based in Munich, Germany. The Flash Pretreatment is well established and is now in operation in a pilot plant in Austria and a demonstration plant in Spain, and the latter also includes Flash Pyrolysis. Stillage recycle and distillation heat conservation are the product of many years of experience by Cemcorp Ltd., of Mississauga, Ontario. The enzyme production system shown incorporates our own research, and also the design of the enzyme production system of Institut Francais du Petrole, Soustons, France. A Flow Chart for a much larger plant which processes 128,000 tons per annum of waste paper to produce 126 cu m ethanol is shown in Section 8.2.2 below. The latter is used for the economic analysis given in Section 9 below.

8.2 Mass Balance

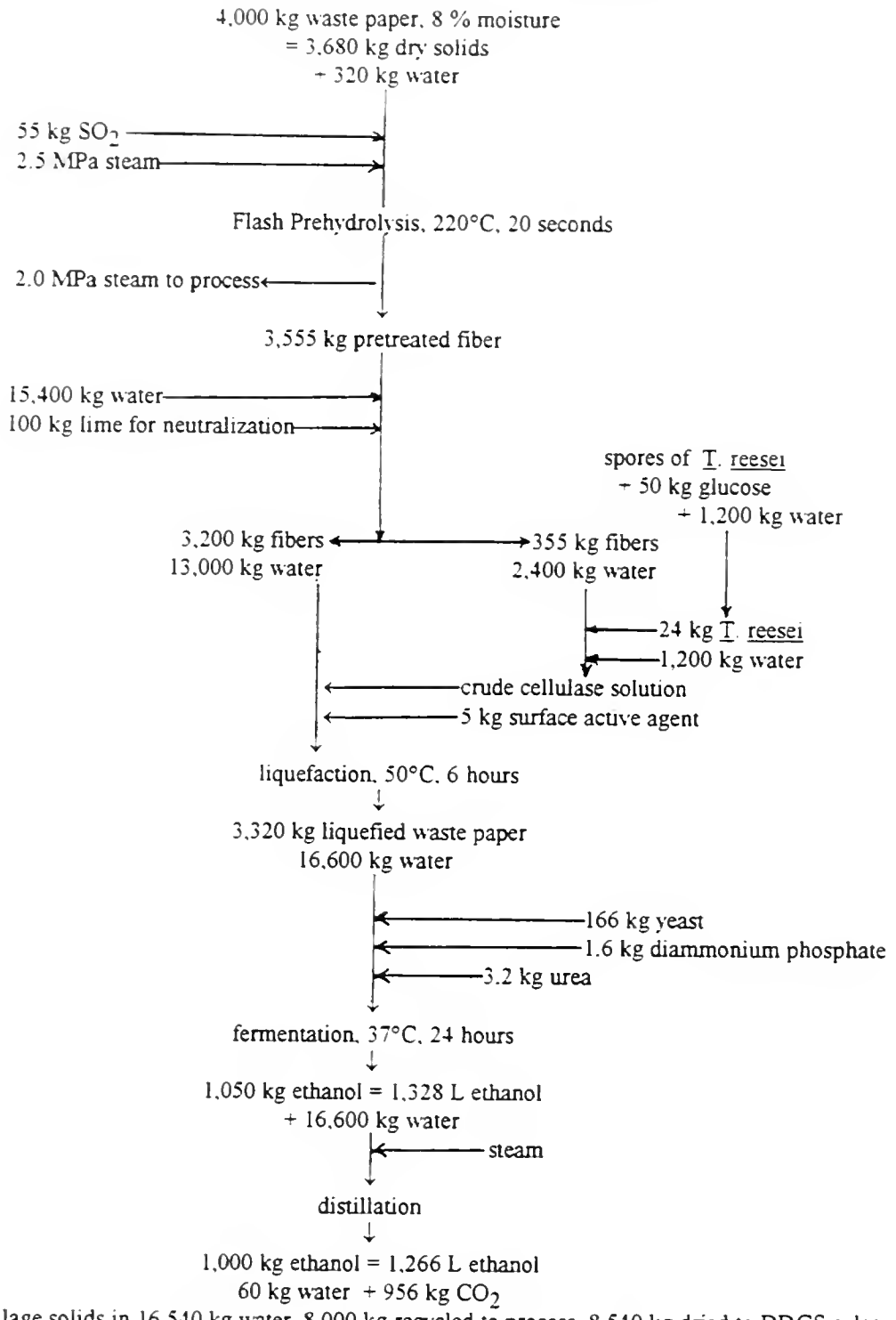
8.2.1 Demonstration Plant Mass Balance

The following chart is a mass balance of a demonstration plant through the process, on a daily basis. The plant receives 4 tons of waste paper containing 8 % moisture per 8 hour day, 5 days per week, 1,000 tons per 250 day year, and produces 1,226 L ethanol/day, a total of 316,000 L/a. In addition to the waste paper, inputs per day include 7,000 kg high pressure steam, 8,900 kg water, 55 kg sulphur dioxide for pretreatment, 100 kg lime for neutralization of the pretreated fiber, 166 kg bakers' yeast and a small amount of nutrients for the yeast. In addition to 1,000 kg ethanol = 1,266 liters, the plant produces 956 kg carbon dioxide. Unused residue is treated by Flash Pyrolysis. The chart on the next page provides data on inputs and outputs on a step by step basis.

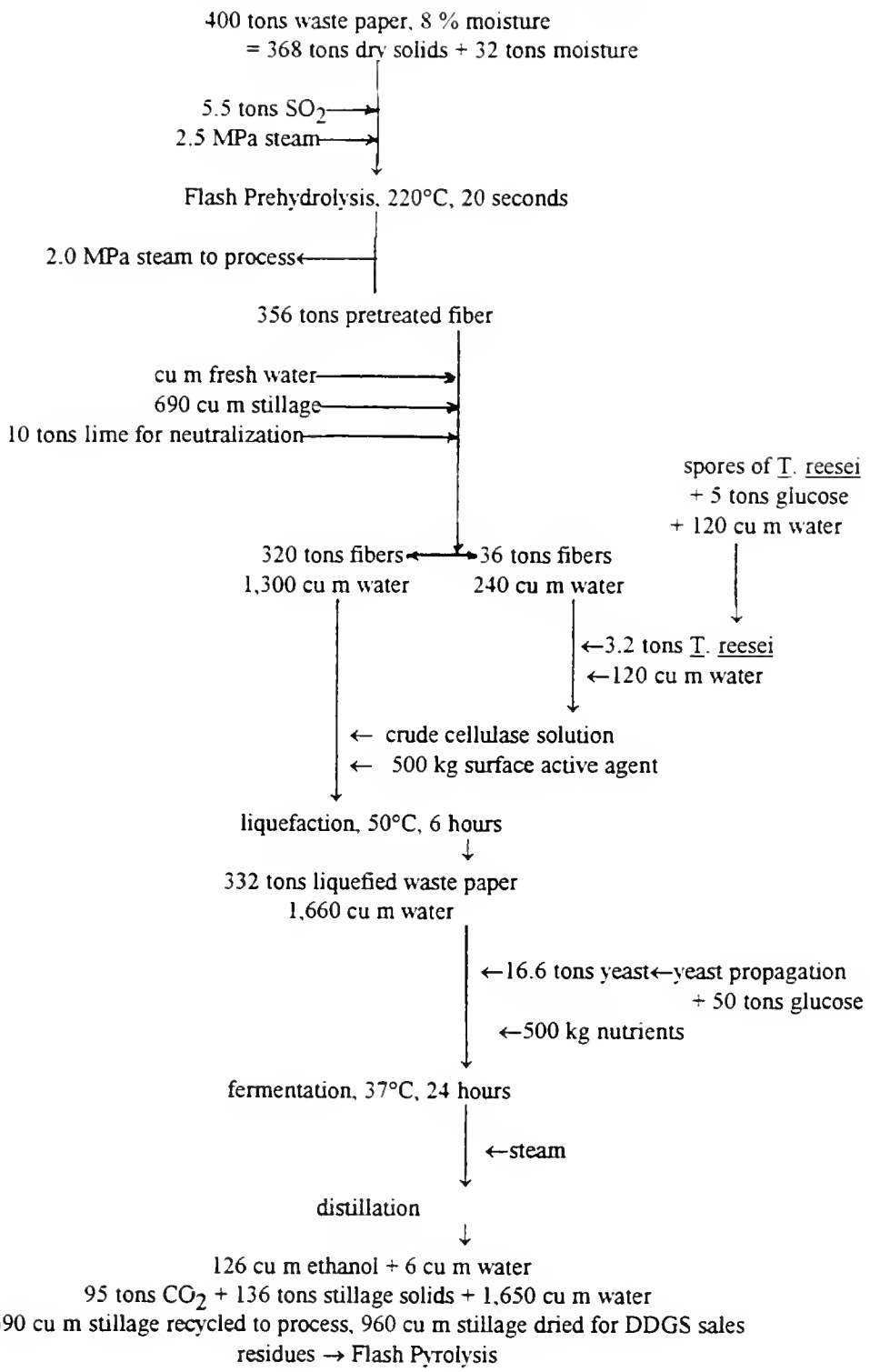
8.2.2 Full Scale Plant Mass Balance

The following chart is a mass balance of a full scale plant, through the process step by step, on a daily basis. The plant receives 400 tons waste paper/day, 7 days/week, 320 days/a, a total of 128,000 tons/a. It produces 100,000 kg ethanol/day = 126 cu m ethanol/day, 40,500 cu m ethanol/a. A difference from the demonstration plant is that the part of the stillage which is not recycled to fermentation is dried and 60 tons/day are sold as dried distillers grains solids DDGS for cattle feed. The amount of yeast needed, 16.6 tons/day, justifies installation of a yeast propagation

DEMONSTRATION PLANT DAILY MASS BALANCE



FULL SCALE PLANT DAILY MASS BALANCE



facility, and this is shown on the mass balance chart. The amount of carbon dioxide produced, 95.6 tons/day is borderline for the installation of a recovery facility. Other inputs are 100 times as great as those shown above for the demonstration plant on a daily basis, and apply 320 days/a instead of 250 days/a. Steam consumption is 7100 tons/day (5.6 kg/L). For pretreatment, 5.5 tons/day of sulphur dioxide are needed, and 10 tons of lime for neutralizing the pretreated fiber. The amount of water in process is 1,660 cu m/day, of which about one-half is recycled stillage, leaving a net intake of 850 cu m net fresh water intake. For enzyme production, 5 tons glucose/day are required.

9. Economic Analysis

9.1 Basis of Estimates

The economic analysis here is based on the full scale ethanol plant for the bioconversion of 128,000 tons of waste paper to 40 million liters of ethanol per year which is described in Section 8.2.2 above. In any economic analysis of an ethanol plant, say from corn, it turns out that the cost of feedstock represents 40 - 60 % of the cost of the ethanol. Here we have a special case where the cost of the feedstock is either zero or negative. In our economic analysis here we will assume zero cost feedstock and calculate a return on investment (ROI) by conventional means. If that proves to be insufficient to attract private capital, we shall calculate what negative feedstock cost ("tipping fee") needs to be charged to waste paper generators for relieving them of the burden of waste paper disposal, while yielding acceptable levels of profit, 20, 25 or 30 %.

Capital costs are based on published data for ethanol plants. We are especially indebted to the study "Ethanol Fuel from Ontario Grain" by Cemcorp Ltd., Misssissauga, Ontario, March 31, 1992 for updating some of these costs; of course our plant configuration differs from Cemcorp's in several important respects, and these are recognized and taken into account in estimating capital costs and operating costs. Revenue for the plant will be based on sales of ethanol and of dried distillers grains solids (DDGS) with no allowance for CO_2 sales. In this economic analysis, the sales price of ethanol at the plant gate is taken as 25 cents per liter. This is higher than Cemcorp's price of 22 cents per liter, but much lower than the value of the ethanol when both provincial and federal tax credits are taken into account.

These tax credits should be divided between producer and distributor and this producer is claiming little of it, perhaps too little. Plant income is then $0.25 x 40 million = $10 million + 19,200 x $104 = $2 million, a total of $12 million.

Capital cost esimates in Table 13 have been estimated in 2 categories, fixed capital and variable capital, the sum of which is the investment required for the plant. Fixed capital includes land and site preparation, structures including buildings and civil works, feedstock receiving and storage, Flash Prehydrolysis, enzyme production, liquefaction, fermentation, yeast propagation, distillation, ethanol denaturing and drying, DDGS drying and other equipment, power house including boiler, substation and other uilities. Total fixed capital totals $40 million, which amounts to $1.00 per annual liter, which is happily in the midpoint of others' estimates. However, others pay scant attention to what we call here variable capital which includes working capital at 1 year's operating cost, engineering and construction management at 2.5 % of fixed capital per year for 3 years, that is 7.5 % of fixed capital, and financing costs at 16 % for one-half of 3 years = 24 % of fixed capital. Total variable capital is $18.85 million, and total investment is $58.85 million, or $1.47 per annual liter. This is higher than the numbers given in the Cemcorp report, which covers only fixed capital costs and does not include working capital or financing costs. Also this plant is more complex than a corn- or grain-based plant because of the need to make the enzyme.

Operating costs include steam at 7 tons per day, electricity, water, sewer charge, glucose, SO_2, maintenance, payroll and depreciation, this last on a 20-year straight line basis. Total daily operating costs are $25,750, or $8.25 million per annum.

9.2 Capital Cost Estimates.

These are given on Table 13, and the total is referred to above.

9.3 Operating Cost Estimates

These are given on Table 14, and the total is referred to above.

Table 13. Capital Cost Estimates

	$million Cdn
1. Land and site preparation	2.0
2. Structures and civil works	3.5
3. Waste paper receiving	1.5
4. Flash Prehydrolysis	2.0
5. Enzyme production	1.0
6. Liquefaction	1.5
7. Yeast propagation	1.0
8. Fermentation, distillation, ethanol drying and storage	14.0
9. DDGS preparation	5.5
10. Power house and utilities	8.0
FIXED CAPITAL	40.0
Working capital, 1 year's operating cost	6.25
Engineering and construction management 2.5 % of fixed capital for 3 years = 7.5 % of fixed capital	3.0
Financing costs, 1.5 years @ 16 % = 24 % of fixed capital	9.6
VARIABLE CAPITAL	18.85
TOTAL INVESTMENT	58.85

Table 14 Operating Cost Estimates, Canadian $

	per ton	per day	per year
SO_2	120	660	211,200
steam, 7 tons/day	7.4	52	16,560
water, 850 cu m/day	(.22/ cu m)	187	59,840
sewer charge		187	59,840
glucose, 35 tons/day	70	2,450	784,000
electric power	.05/kwh	2,520	806,400
maintenance, 2 % of fixed capital		2,500	800,000
payroll, 100 @ $35,000/a		10,938	3,500,000
depreciation, 5 % of fixed capital		6,250	2,000,000
OPERATING COSTS		25,750	8,250,000

9.4 Profitability

The return on the investment of $58.85 with sales of $12 million and costs of $8.25 million is meager, 6.4 %. This is not enough to attract private investment. To obtain higher ROI either investment needs to reduced or income increased. Reduced investment may come from government subsidies in some form such as, for example, loan guarantees or direct grants. Guaranteed loans would reduce financing costs, from $9.6 to $6.4 million, thereby raising ROI from 6.4 % to 6.6 %, not enough to attract investors. Increase of income has a much greater effect. Income increases may be achieved by a higher selling price for ethanol or by charging a tipping fee for taking waste paper. A higher selling price for ethanol is certainly justified by its high octane number - it is used primarily in premium grades of motor fuel - and by the extensive provincial and federal subsidies. At a plant gate selling price of 30 cents/L of ethanol, a conservative figure still below its value, ROI becomes 9.8 %. We have calculated the tipping fee required to raise ROI to a satisfactory level, NOT taking into account any increase in the plant gate price for ethanol, and find that 20 % ROI requires a tipping fee of $62.00/ton waste paper, while for 25 and 30 % ROI tipping fees of $85.60 and $108.60 are required. These tipping fees are substantially below charges by Metropolitan Toronto which may reach as high as $175.00/ton.

A coincidence of 2 factors, government guaranteed loans to reduce financing charge by $2.4 million leaving investment at $56.45 million, and an ethanol price of 30 cents per liter to increase annual sales to $14 million, ROI of 20, 25 and 30 % would require tipping fees of 43, 65 and 87 $/ton. Tipping fees at this level may well attract generators of waste paper such as banks to participate in enterprises for bioconversion of waste paper to fuel ethanol.

10. A Better Best Process for Bioconversion of Waste Paper to Ethanol

The technology described in this report is sufficiently advanced to form the basis for a demonstration plant such as that described in Section 8.2.1 above or under very favourable circumstances a full scale plant such as that described in Section 8.2.2 above. However, there are remaining deficiencies in the technology which should be the subject of continuing research. These are cellulase production, β-glucosidase supply and xylose fermentation. To advance the technology in these areas would give rise to a better "best" process (compare Section 4.6 above). The first 2 of these would reduce processing costs, while xylose fermentation would increase ethanol yields significantly.

10.1 Cellulase Production

The principal advances represented by our research findings (Appendices A-6, A-7 and A-8) are (i) the use of inexpensive cellulase inducers such as waste newspaper and whole wheat flour; and (ii) the finding that whole wheat flour is not only an effective inducer but is also an adequate source of nutrients for both cellulase production and fermentation. The particular roles of bran and of sorbose also add to existing technology. However, the cellulase enzyme we produce is far too dilute. Iogen claims higher concentration, while IFP Soustons claims enzyme concentrations 15 times higher than we experience, that is 22,000 FPU/L compared to our result of 1,400 FPU/L. In terms of capital and operating costs, higher enzyme concentrations have significant advantages. This is an area to which we are giving increaig attention, and it is now at the top of our research priorities. We expect to be able to duplicate IFP's result during this calendar year 1992. We understand what needs to be done. The major difficulty is around the non-availability of minor equipment, which when not available becomes a major difficulty. However, we shall overcome.

10.2 β-Glucosidase Supply

We must get away from using purchased cellobiase (β-glucosidase). There is some discussion of this problem in Section 6.4 above. We have 3 quite different approaches. In order of increasing difficulty these are: to employ cellobiose-fermenting yeasts in fermentation; to identify a

microorganism which excretes β-glucosidase, and to grow it and induce β-glucosidase production similar to cellulase enzyrne production; and thirdly, to apply molecular biology, transferring the β-glucosidase gene from a different organism into common bakers' yeast. We have already identified, and published papers describing several cellobiose-fermenting yeasts, and have experienced good results from time to time.There are, however, certain difficulties. For one, the saccharification-fermentation strategy that is incorporated in our preferred process requires a yeast that grows and ferments well at somewhat elevated temperatures, at least 37°C. Also these yeasts already tend to be slower in growth and fermentation than common yeast, so a non-optimal temperature is a handicap. To overcome this, we are planning to used mixed cultures, in which bakers' yeast would be given a head-start of say 6 to 12 hours and then the special yeast added. This should be easy to accomplish.

The de novo synthesis of β-glucosidase is likely to be difficult. There have been rare occasions in the production of cellulase when we have also made a reasonable level of cellobiase (see, for example, Figure 12, p 61). It is conceivable that careful, properly oriented study of the conditions of cellulase synthesis by T. reesei would result in a procedure for production of β-glucosidase. This probably would involve other inducers, Also production of cellobiase, like production of cellulase, is an aerobic process, strongly inhibited by glucose. The glucose can be removed by fermentation; however, fermentation is an anaerobic process. We therefore face a dilemma in controlling air or oxygen supply to cellobiase synthesis.

Another different strategy involves surveying the literature and the culture collections for microorganisms that produce, or can be induced to produce, cellobiase. This needle-in-the-haystack approach would be feasible in a large research organization but not in our tiny laboratory. While we plan to pay attention to all of these possibilities, our main research effort will focus first of all on cellobiose-fermenting yeasts. We have now acquired several of these and they are being evaluated for their effectiveness in our process for bioconversion of waste paper to ethanol.

10.3 Xylose Fermentation

Waste paper stocks normally contain 10 - 15 % xylan. Upon saccharification, xylose is produced. Xylose is not fermented to ethanol by

common bakers' yeast. We have several yeast species which, upon proper preparation, ferment xylose efficiently to ethanol. The preparation of such yeasts for efficient xylose fermentation involves recycling the yeast after fermentation to fresh hydrolysate, and repeating the recycling 4 - 10 times. We have shown that such repeated recycling of the yeast in a stressing medium results in transition from the common haploid form to a diploid form. These diploid yeasts are larger and more robust, and are particularly adept at fermenting xylose. Among the yeast species which are most effective in xylose fermentation are the recycled strains (R strains) of Candida shehatae and Pichia stipitis.

So what is the problem? The problem is that xylose fermentation to ethanol requires the presence of oxygen (or perhaps some other hydrogen acceptor) whereas glucose fermentation to ethanol is anaerobic. We have succeeded in fermenting glucose and xylose together in wood hydrolysates and hope to do so in waste paper hydrolysates. The trick is to control air addition to match closely the amount of xylose present at any time, a condition we have called semi-aerobic. This means a fairly large flow of air at the beginning of fermentation tapering off as the xylose is consumed.

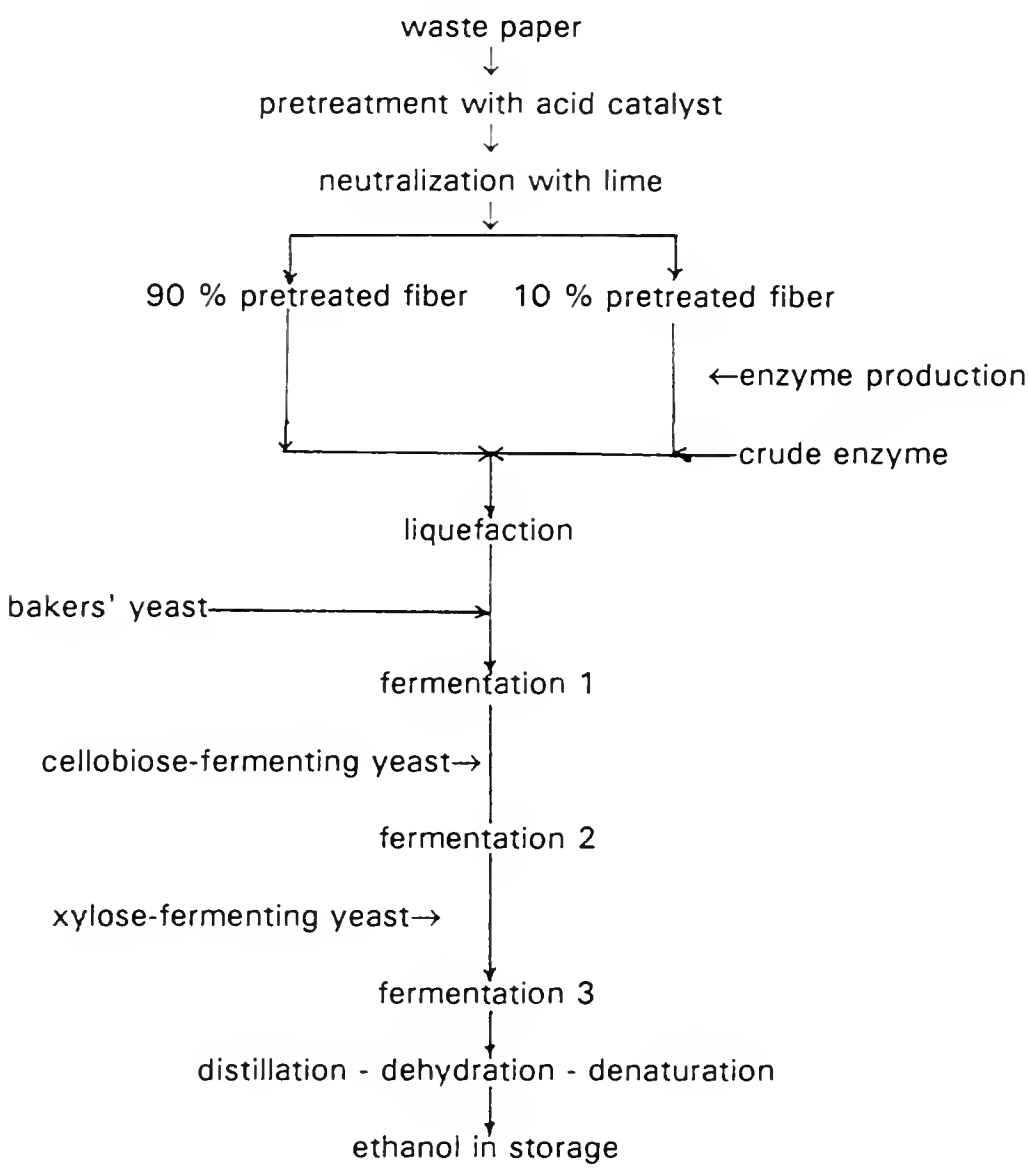
The optimum oxygen/xylose ratio for efficient xylose fermentation has not been identified, and this is of some importance. Too much air would mean inefficient glucose fermentation with biomass production instead. Too little air would leave the xylose unfermented. Rather straighforward experiments using a dissolved oxygen recorder-controller would settle this question. Unfortunately, this equipment is not available for our use. We plan to continue research on xylose fermentation in waste paper hydrolysates as we can make people's time available for this purpose.

10.4 A Better Best Process for Bioconversion of Waste Paper to Ethanol

In Section 4.6 above a "best" process for bioconversion of waste paper to ethanol was described. It included the steps of liquefaction-fermentation, as well as enzyme production. In light of our new knowledge we would now add pretreatment with acid catalyst, the use of cellobiose-fermenting yeasts, the use of xylose-fermenting yeasts, and the recycling as far as possible of all of these yeasts, both common and exotic. Because of the heavy slurries in fermentation with residues from the paper suspended in the medium, such recycling depends on the development of suitable screens. Also the whole set of concepts would be much more practical in a

cascade fermentation system (see p 122 in "Biotechnology of Biomass Conversion"), where successive fermenters would have different yeasts: common yeast, cellobiose-fermenting yeast and xylose-fermenting yeast. Since each of these requires somewhat different conditions, air flow for example, this becomes practical in a cascade system, in which fermenting medium flows by gravity from each fermenter to the next._Table 15 charts a better process for bioconversion of waste paper to ethanol

Table 15 A Better Process for Bioconversion of Waste Paper to Ethanol



The cascade system may be expanded at the front end to include fungal growth and cellulase enzyme formation. In practice it is not at all necessary, or even desirable, to separate and purify the enzyme, on the contrary the crude enzyme is more effective in saccharification and contains desirable nutrients. Hence a 6-fermenter cascade would have these vessels:

1. fungal growth
2. enzyme production
3. liquefaction
4. fermentation of glucose
5. fermentation of cellobiose
6. fermentation of xylose

the contents of each vessel flowing by gravity to the next, with adjustment of temperature, pH, additives and air flow. Ten percent of the prehydrolysed fiber would be added to vessel 2, and the other 90 % to vessel 3, batch-wise rather than continuously. This simple linear system could well form the basis for the design of a pilot plant or demonstration plant to be built in Ontario, if the will can be found to do so.

11. Technology Transfer and Entrepreneurial Prospects

11.1 Ontario and Canada

There are no industrial operations in Ontario, or in Canada, which make ethanol from waste paper. The Commercial Alcohols plant, based on waste steam from the Bruce nuclear station, makes 15 million liters of fermentation ethanol from corn, some of which goes to UCO Petroleum's motor fuel. Mohawk in Manitoba makes 10 million liters/a of fermentation ethanol from grain, added to motor fuel in their own gas stations. Poundmaker, in Saskatchewan, has a grain-based ethanol plant making 10 million liters/a, at least some part of which goes into Mohawk's gasohol. Tembec, in Quebec, makes 16 liters of industrial alcohol from spent sulphite liquor. Total production of the Canadian ethanol industry, all except Tembec's from corn or grain, is about 50 million liters/a. Canadian Agra, which has extensive agricultural operations in Ontario, has been active in lobbying for the removal of the federal excise tax on ethanol in motor fuel, and is now planning a corn-based fuel ethanol plant variously reported to be 80 or 100 million liters/a, thereby more than doubling Canadian capacity.

Ontario has a few farm-based alcohol distilleries which make fuel ethanol for their own purposes - one farmer told me that he uses 30 % alcohol of his own making in motor fuel for his mobile equipment - and similar interest has been expressed by groups of farmers in Northumberland and in Cornwall, who are prepared to invest in an alcohol plant but need guidance and encouragement. United Cooperatives of Ontario has gone into partnership with Sun Oil to form UCO Petroleum. They are supplying 21 gas stations in south-western Ontario with corn-based ethanol, mostly as of now bought from Commercial Alcohols. The ethanol is used at a 5 - 10 % level in midrange and premium gasoline. Their total ethanol consumption now is 1.5 million L/a. They plan to expand to many more stations "until such time as one of the major oil companies says 'enough is enough!'" Their main limitation is supply of ethanol. However, there was no indication that UCO Petroleum is considering making its own ethanol.

The prospect for Ontario appears to be a number of small farm-based ethanol plants supplying ethanol to 1 to 25 farms, on a cooperative basis; perhaps a fermentation ethanol plant to supply UCO Petroleum; and Canadian Agra's large corn-based ethanol plant.

In spite of many nibbles, no entrepreneur has expressed serious interest in investing in a waste paper-based ethanol plant. This writer has addressed many meetings, including the Ministry of the Environment's Technology Transfer Conference late in 1991 and the Ontario Ministry of Food and Agriculture's Ethanol Conference in 1992, he has met with many interested parties, and he has published several papers on this subject, as well as appearances on TV and on radio. Noone in Ontario has come forth with a dollar. He still has some lingering hope that the St. Lawrence fermentation plant and distillery may be used as a 10 - 20 million liter/a demonstration plant for bioconversion of waste paper to ethanol, but as time passes, this hope becomes dimmer.

Why so little interest? The fact is that generators of waste paper and truckers have devised ingenious schemes for disposal at less than the official tipping fee, the main method being export to the US: cross-border shopping in reverse.

We are also discouraged by the low level of research on bioconversion of waste paper to ethanol. Aside from our laboratory in the University of Toronto, Stake Technology and Iogen appear to be the only research centers interested in any aspect of this problem. The advances Iogen has made in enzyme synthesis represent a very important advance arising from Ontario research and business, yet the cellulase enzyme they sell is made in the US. No other research laboratories in Ontario have surfaced. (Our research on pretreatment of waste paper ahead of its conversion to ethanol was carried out in part in the laboratory of Professor Esteban Chornet in the Universite de Sherbrooke, as described in Appendix A-4, an example of interprovincial cooperation).

There is a great need in Ontario for research, pilot plant, demonstration plant and full scale plant for bioconversion of waste paper to fuel ethanol, in sum for the establishment of a biofuels industry. But that will probably happen elsewhere, most likely in Europe.

11.2 Europe, Japan and Other Countries

Significant amounts of money are now being spent on planning a demonstration plant for bioconversion of waste paper to fuel ethanol in

Western Europe, and a full scale ethanol from waste paper plant in Eastern Europe, these being essentially the 2 plants described in Sections 8.2.1 and 8.2.2 and Section 9. Novel elements which will be used in these plants are in operation in demonstration plants in Austria and Spain. Europe is strapped for land for landfill of its mountains of garbage, especially waste paper, which is generated at the rate of 150 million tons yearly in OECD countries, and also is highly dependent on imported motor fuel. The prospect of relief of these 2 major problems has attracted both government and private investment. In addition, Eastern Europe has a serious problem of redundancy of workers, and of old-fashioned industrial facilities. The promise of new employment opportunities at the same time as meeting environmental demands is also an important factor in bureaucratic decision-making there. The prospects for the establishment of a major new industry in Europe based on bioconversion of waste paper to fuel ethanol are bright indeed. We and other Ontario companies believe the technology we have developed is marketable, and our participation in European developments will confirm our belief.

Japan imports all of its oil. There are several consortia of industry and government in Japan devoted to the study of alternative motor fuels, and ethanol in particular. Their research is based on sugar, lignocellulosics and grass. Several elaborate and expensive pilot plants have been built, and there are several university centers of research on alcohol from lignocellulosics. This writer has met many of the leading research people, both in Japan and here. So far, however, there has been no interest in MSW or waste paper.

In the US, research and pilot plant attention is turning increasingly to MSW. Leading research centers are TVA, Muscle Shoals, Alabama and the National Renewable Fuels Laboratory, Golden, Colorado. These, and some other laboratories, have begun to publish the results of laboratory and pilot plant research on ethanol from MSW. Waste paper as such is not being studied, nor are any industrial facilities contemplated. This is surprising, since there are many MSW separation plants in the US which produce a light fraction enriched in paper. These could serve as a source of waste paper in the form of RDF or some further enriched fraction as feedstock for a bioethanol plant. Some of the companies involved have expressed an interest verbally in our research here, but so far no money. The only outlet for RDF is incineration for generation of power in one form or another. Incineration is increasingly frowned upon by environmentalists - it is illegal in

Ontario - and we may see a bioethanol industry using RDF as feedstock developing around these MSW separation plants in the US. This is important in the US, where one-third of their oil is imported from regions which are politically unstable. US MSW could supply enough motor fuel to displace *all* Middle Eastern oil.

The largest consumer of fermentation ethanol in motor fuel is Brazil, where it now exceeds 20 % of their gasoline; in Brazil it is all made from cane sugar or molasses. Aside from Canada, Europe, Japan and the US, no serious interest in fermentation ethanol from MSW or waste paper has come to my attention.

12. Conclusions and Recommendations

12.1 Conclusions

1. The segregation and collection of all waste paper in Ontario would reduce the volume of MSW going to landfill by 40 - 50 %. If a commercial MSW fractionation system such as is common in Europe and the US were in place, the reduction in volume would be 80 - 90 %. The waste paper so diverted would be used to make valuable marketable products such as ethanol.

2. There is enough MSW-paper in Ontario to displace 10 % of gasoline upon bioconversion to the environmentally friendly, high octane, non-petroleum motor fuel ethanol. Improvements in air quality, lessening of crop damage from ground level ozone and better human health can be expected.

3. Potential markets for ethanol in Ontario are large, annual motor fuel consumption being around 12,000 million liters. Substitution of 10 % of this by ethanol would provide a market of 1,200 million liters per year. To supply this market would require a new industry of 20 - 24 full size distilleries employing about 2,500 workers permanently, on site, and an equal number occupied in the supply of waste paper, and about 20,000 man years of employment in construction. Investment of about $60 million in each plant would amount to $1.2 -1.5 billion.

4. A best process for bioconversion of segregated waste papers, and of the light fraction of mechanically separated MSW, usually designated Refuse Derived Fuel (RDF) because of its high calorific content, is detailed in this Report. The process can be epitomized as enzymatic liquefaction followed by saccharification combined with fermentation by common bakers' yeast. Enzymatic saccharification is preferred to acid hydrolysis of waste paper because the conditions are milder and yields of ethanol are higher. The necessary enzymes can and should be made on site. By this best process, yields of ethanol of 350 - 400 liters/ton of waste paper, or 200 liters/ton of RDF were obtained. These are 70 - 80 % of the ethanol potential based on fermentation by bakers' yeast.

5. The yields just quoted were obtained without any pretreatment ahead of liquefaction. A very brief steaming at 200°C with 0.5 % sulphuric acid catalyst raised yields of ethanol from waste paper to 460 liters/ton, 90 % of the theoretical potential based on cellulose content and the use of bakers' yeast. Pretreatment with acid catalyst as just described has the advantages of sterilizing the waste paper, thereby avoiding bacterial contamination, and shortens fermentation times, as well as these higher yields.

6. The technology described in this Report is sufficiently advanced to form the basis for a demonstration waste paper to ethanol plant and, under favorable circumstances such as exist in Eastern Europe, a full scale plant. The technology is exportable, and plans are in progress to apply it to such enterprises in Europe, both East and West.

7. Flow Charts and Mass Balances are presented here for 2 sizes of ethanol plants, a demonstration plant accepting 4 tons/day, 1,000 tons/year of waste paper, and a full scale plant receiving 400 tons/day, 128,000 tons/year of waste paper, showing all inputs, process conditions and products.

8. The Mass Balance of the full scale plant was used for economic analysis, which gave total investment of $58.5 million including fixed capital, working capital, engineering and management during construction and financing costs. Operating costs, including maintenance and depreciation, but accepting waste paper at zero

cost, came to $8.25 million/year. Revenue, based on sale of 40 million liters of fuel ethanol at 25 cents/liter plus sale of Distillers Dried Grains Solids, amounts to $12 million/year. This gives a crude Return on Investment of only 6.4 %, not enough to attract private investors. To raise ROI to an acceptable level, 20, 25 and 30 %, requires tipping fees of $62.00, $85.60 and $108.60/ton of waste paper. An ethanol price of 30 cents/liter, which is more than justified by provincial and federal subsidies for ethanol in gasoline would reduce these tipping fees to $43, $65 and $87/ton. A smaller plant would require larger tipping fees for equivalent profitability. For example, a plant of 20 million liters ethanol annual capacity would require a tipping fee of about $60 - $65/ton for 20 % ROI. These are significantly lower tipping fees than present municipal tipping fees, and may well attract generators of waste paper such as banks to become sponsors or partners in enterprises for the bioconversion of their waste paper to ethanol.

9. Capital and operating costs for a waste paper to ethanol plant may be reduced significantly by partnering with a nearby enterprise which has available excess low pressure steam and/or electricity, such as a pulp mill or nuclear plant. Also a partner which can make more profitable use of the residues from this plant may help the economics and simplify the design.

10. Economic success depends on the use of enzymes made on site rather than purchased enzymes. In the corn syrup-glucose industry, which has many parallels, enzyme prices are low enough that most of the industry uses purchased enzymes rather than making their own. This Report includes a detailed description of the synthesis of the main cellulase enzyme using waste newspaper or other inexpensive carbon sources. In our Flow Chart for ethanol from waste paper we show 10 % of the pretreated waste paper being diverted to enzyme production. Laboratory results indicate that this figure may be much too high, and that perhaps as little as 5 % of pretreated waste paper would be needed for enzyme production.

11. A better best process, described here, would include acid catalysed pretreatment, xylose fermentation and cellobiase supply by the use of other yeasts, named in this Report. By conducting fermentation in a

cascaded series of fermenters, the application of these exotic yeasts, would be relatively simple. Xylose fermentation would raise yields by about 10 % higher than obtainable by the use of bakers' yeast, and the use of cellobiase containing yeasts would lower the cost of enzyme.

12. Motor fuel containing 5 - 10 % ethanol, marketed in southwestern Ontario by UCO Petroleums, and a small volume in Mohawk's northwestern gas stations contain only corn- or grain-based ethanol, and the ethanol plant being planned by Canadian Agra will be corn-based. In addition, there are a few small farm-based distilleries, the products being used in mobile equipment. Specific steps to encourage the building of a biofuels-from- waste paper industry are given in the Recommendations.

12.2 Recommendations

1. The bioconversion of Ontario's waste paper to motor fuel ethanol requires the establishment of a new 20 - 25 plant industry, with investment of $1.2 - 1.5 billion. The promise of such enormous industrial activity, with accompanying employment and environmental benefits, demands serious attention. Ministries of the Environment, Energy, Agriculture and Food, and Transportation should join to encourage the development of this new industry.

2. Research and development in bioconversion of waste paper to fuel ethanol needs to be substantially increased to a level commensurate with its environmental and industrial importance. Existing facilities are inadequate. A proper pilot plant capable of solving problems of feedstock, process, microbiology and engineering is required. For example, the search for special strains or mutants of fungi, yeasts and bacteria which have beneficial characteristics should be a continuing endeavour. Consideration might be given to building on Iogen's pilot plant, or St. Lawrence Reactor's pilot plant, neither of which is adequately equipped now. Adaptation and expansion may take less time than a grass roots facility, however that is far from certain, and needs a proper assessment. The new pilot plant should be equipped to test a range of modern technologies for bioconversion of waste paper to fuel ethanol: simultaneous saccharification and fermentation, enzyme production and use, extractive fermentation,

cracked corn dehydration of 80 % (or less) ethanol concentration, cascade fermentation, xylose-fermentig yeasts, cellobiose- fermenting yeasts, immobilized yeasts for very rapid fermentation, and stillage recycling and disposal.

3. The exportability of the technology described in this Report is established by the European response. A continuing effort to expand the adoption of this technology in the US, Europe and Japan is justified and needs to be pursued.

4. Waste paper needs to be considered as much a resource as, say, spent sulphite liquor, and should be collected by waste paper generators and converted to ethanol. A consortium of banks should be encouraged to follow this route with their own waste paper. Large financial and industrial conglomerates should be encouaged to follow this pattern. Similarly, fibres in effluents from pulp mills should be treated as a source of ethanol for energy in their own operations.

5. Consideration should be given to dedicated biofuel plants based on specific segregated feedstocks such as waste paperboard packaging. Such papers have characterisitics which may benefit from modified process procedures and may do better in terms of yield and costs in such dedicated plants than when included in mixed waste paper feedstock.

6. Since the segregation and collection of waste paper is estimated to reduce the volume of MSW to landfill by 40 - 50 %, while an MSW fractionation system such as is common in Europe and the US promises to reduce that volume by 80 - 90 %, engineering studies of the costs involved in the installation of such MSW fractionation systems need to be carried out. Such a Materials Recovery Facility would produce more marketable products, and lends itself to integration with other resource recovery systems.

7. Research on improved processes for production of cellulase enzymes is a matter of some urgency, to be encouraged by the Ministry.

8. The identification or development of a yeast strain which is a good fermenter at 40°C or above should have a high priority in the research program. Fermentation by such a strain should be accompanied by vacuum distillation of the ethanol as produced.

9. Fermentation of xylose requires oxygen (or air): too little results in poor xylose fermentation, while too much results in growth of yeast at the expense of ethanol production. To optimize xylose fermentation, we need to know the proper oxygen to xylose ratio. This is a specific piece of information which should be the subject of an important item in the research program.

10. The pretreatment reported here was catalysed by sulphuric acid. The use of gaseous SO_2 or CO_2 as the acd catalyst would have many advantages, and should be studied.

11. The strategy of how feedstock is added, and perhaps of how enzyme is added, to the saccharifying medium needs to be studied for higher ethanol concentrations, thereby reducing distillation costs.

APPENDIX A-1 GRANT APPLICATION EXTRACTS
(terms of reference)

Ministry of the Environment / Ministère de l'Environnement
Ontario

Ministry Use Only Réservé au ministère	
Proposal No. Proposition n°	Project No. Projet n°

Principal Investigator Chercheur principal	Affiliation Affiliation
MORRIS WAYMAN	UNIVERSITY OF TORONTO

1. Abstract (For Full Study) Résumé (de l'étude)

This project is directed to reducing the volume of MSW now going to landfill, and the bioconversion of its paper content to a marketable product, the environmentally benign motor fuel alcohol (ethanol). The pending closure of the St. Lawrence Starch 20 million L/year distillery in Mississauga provides an oportunity to move this alcohol plant to the downsview separation plant or equivalent site, and to use the MSW paper as feedstock for alcohol production. At 200 L/tonne, this distillery alone would remove 100,000 tonnes from the MSW stream to landfill. We propose to apply to MSW paper our extensive experience in bioconversion of cellulose to alcohol. Our process begins with enzymatic saccharification of cellulose, the largest component of the paper, followed by fermentation to ethanol. Our proposal includes study of a process for in-plant low-cost enzyme production, the target being to bring the cost of cellulase enzymes to the same level as commercial starch-saccharifying enzymes.

2. Objective(s) Objectif(s)

1. To obtain a representative sample of the light fraction of mechanically separated MSW and, by analysis, determine its potential for saccharification and for production of alcohol.
2. To saccharify the paper component of separated MSW (MSW paper) quantitatively, to measure volume reduction, and to make a mass balance of the process.
3. To convert MSW paper to alcohol by the best process, and to measure yields and enzyme requirements.
4. To study processes for on-site manufacture of cellulase enzymes.
5. To prepare a report which summarizes the results of this work, and considers the economic and technical factors in scale-up.

3. Milestone Definition and Schedule (For Full Study) Définition et calendrier des étapes (de l'étude)

Milestone No. Étape n°	Description Description	Anticipated Date of Completion Date prévue de la fin de l'étape
Assumed	starting date July 1, 1990.	
1.	Objective 1. Analysis of representative samples of MSW paper, and interpretation	Oct 1, 1990
2.	Objective 2. Measurement of volume reduction by MSW paper saccharification	Dec 31, 1990
3.	Objective 3. Quantitative saccharification and fermentation of MSW paper	Jun 30, 1991
4.	Objective 4. Enzyme production processes	Mar 31, 1992
5.	Objective 5. Report production	Jun 30, 1992

4. Study Description Description de l'étude

Year 1 of Study 1re année de l'étude

Description Description

Obtain from several sources representative samples of mechanically separated light fractions or paper fractions from MSW, analyse for cellulose and other components, and calculate their potential for alcohol production. Saccharify the MSW paper and measure volume reduction. Quantitative production of alcohol from MSW paper by saccharification and fermentation, separately and simultaneously.

Anticipated Results Résultats prévus

The data so obtained will be used to provide a basis for estimation of MSW volume reduction by this process, and the amount of alcohol product to be expected. It will identify areas of difficulty and problems to be anticipated in scale-up to a substantial demonstration plant (20 million liters/year). It may identify the better sources of MSW paper.

Year 2 of Study 2e année de l'étude

Description Description

Fine tuning of the above process will continue, but most of the year's effort will be devoted to enzyme production and application. These enzymes are produced by moulds of which two, Trichoderma reesei and Aspergillus niger, are presently used commercially for production of these enzymes. Our experience with the benefits of immobilization of such biocatalysts will be applied to these studies.

Anticipated Results Résultats prévus

Our target is to bring the cost and effectiveness of cellulase enzymes to the same level as starch-saccharifying enzymes. We have faith that this can be done by using our immobilization techniques. The results of this year's work will tell us how close that goal may be approached. A report summarizing the work of these two years will be produced, which will include the laboratory findings, our

Year of Study année de l'étude

Description Description

interpretation of them and their implications for the economic and technical factors of scale-up, an appreciation of potential markets for the gasohol product, and policy implications.

Anticipated Results Résultats prévus

Ministry of the Environment / Ministère de l'Environnement
Ontario

Ministry Use Only Réservé au ministère	
Proposal No. Proposition n°	Project No. Projet n°

Principal Investigator Chercheur principal	Affiliation Affiliation
MORRIS WAYMAN	UNIVERSITY OF TORONTO

5. Relevance and Potential for Application Pertinence du projet et possibilités d'applications

What will this project contribute to the Ministry of the Environment's research needs?
Comment le projet répondra-t-il aux besoins en recherche du ministère de l'Environnement?

This proposal responds to the Ministry of Environment's research needs LS05 Ways and Means of Reducing the Volume of the Municipal Solid Waste Stream, and LS02 Methods for the Treatment of Wastes and Waste Products. The continuing disposal of MSW in landfill is generating political difficulties in Ontario municipalities, and is increasingly expensive as MSW is hauled over greater distances. The expected life of major landfill sites is nearing an end. The significant reduction in MSW volume to be achieved by our process would extend landfill site life, while conversion of a substantial fraction of MSW to a marketable product would relieve its present economic burden. Further, the use of petroleum-based motor fuel has contributed to the generation of smog over Ontario cities. Extensive experience has shown that the use of gasohol, an alcohol-extended gasoline, in the US, Brazil and elsewhere, has provided relief from smog. The present proposal addresses these environmental concerns.

Describe potential for implementation of results, potential users and time frame for application of results by users.
Décrivez les applications possibles des résultats du projet, indiquez les usagers éventuels et fournissez le calendrier de ces applications.

Potential stakeholders in the implementation of our process include operators of fleets transporting MSW to landfill, manufacturers and marketers of alcohol, paper recyclers and the Province of Ontario with its extensive motor fleet. The government of Manitoba has announced that it plans to use gasohol in its fleet of 2800 vehicles. Colorado and other US states have mandated the use of gasohol in the winter months when smog is serious. We can expect that their experience will stimulate similar regulations in Ontario.
Application of the results of this proposal can be very soon. The pending closure of the St. Lawrence Starch Company distillery in Mississauga should cause the Ministry of the Environment to give consideration to the acquisition of that plant and moving it to the Downsview separation plant in anticipation of the manufacture of gasohol from MSW paper. Other stakeholders will act as soon as they are convinced that the technology is sound, and that the political climate in Ontario is favourable.

Potential Marketability – Please describe if applicable
Possibilités de commercialisation – Donnez-en une description le cas échéant.

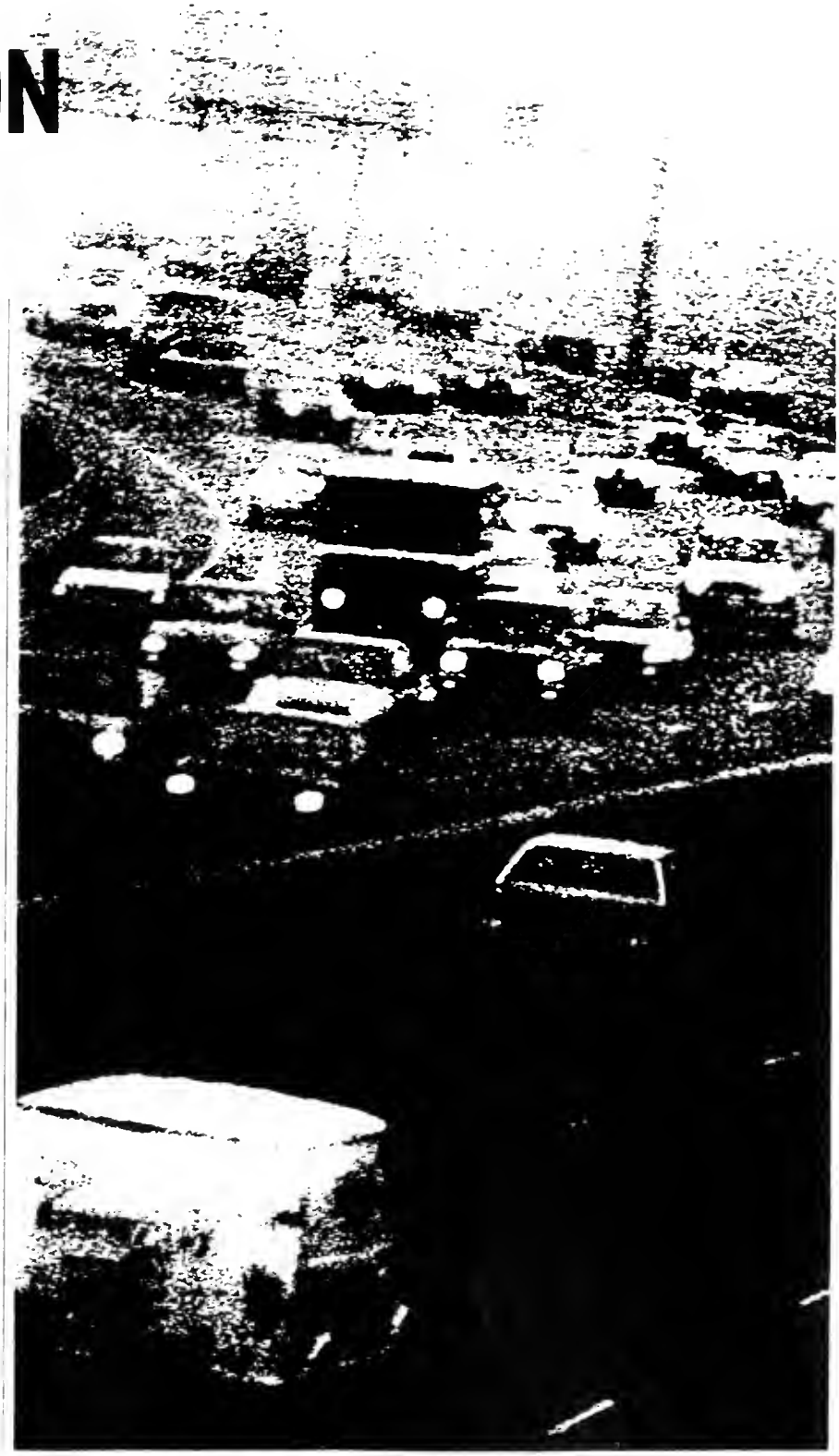
Potential markets for gasohol in Ontario are large, annual motor fuel consumption being about 12,000 million liters. If that were extended by alcohol to the extent of 10%, the alcohol market for that use would be 1,200 million liters per year. The proposed Downsview distillery at 20 million liters per year would supply 2% of that. At 200 liters/tonne of MSW paper, this distillery alone would remove 100,000 tonnes from the MSW stream to landfill. Ontario MSW paper fully utilized in this way would provide nearly 40% of Ontario-wide gasohol.

Specific investors could include transportation companies, paper recyclers, alcohol producers and marketers such as Commercial Alcohols Limited and Mohawk Oil, as well as publicly owned provincial and municipl motor fleets.

APPENDIX A-2 "Motor Vehicles and Air Pollution,"
Alan Goodall, Statistics Canada, 1992

MOTOR VEHICLES AND AIR POLLUTION

by Alan Goodall



Motor vehicles in general, and automobiles in particular, continue to be a major source of air pollution in Canada. Although emissions have been regulated since the early 1970s, motor vehicles remain the largest source of several pollutants that adversely affect human health and the environment. Most Canadians, particularly those in urban areas, continue to be exposed to potentially harmful levels of these pollutants.

Despite a growing number of vehicles, the average concentrations of these pollutants has declined since the 1970s — largely as a result of motor vehicle emissions regulations. However, unless new strategies for emission reduction are adopted, current pollution levels are expected to increase as the motor vehicle fleet continues to grow.

Consistent with recommended emission reduction strategies, programs to deal with anticipated emission problems are now in the planning or early implementation stages. Among others, current efforts include attempts to further increase the use of alternatives to private motor vehicle transportation, and to further reduce the emissions output of individual vehicles.

Emissions and effects

Canadian federal and provincial agencies have been jointly monitoring national air pollution levels since the 1970s. Following the passing of the Clean Air Act in 1971 and the subsequent introduction of emission standards and controls for industry and motor vehicles, Canadian air quality has improved notably. However, because most Canadians (76%) live in or near major urban centres and use motor vehicles, they are still exposed to potentially harmful concentrations of some air pollutants.

Four common air contaminants are carbon monoxide, nitrogen oxides, hydrocarbons, and ground-level ozone. Together, in high concentrations, these four pollutants are known to affect pulmonary function, suppress the immune system, and have toxic and carcinogenic effects. They can also suppress or otherwise affect vegetation growth, corrode metals, fade fabric dies, degrade rubber products, textile fibres and polyurethanes, and contribute to the formation of acid rain. In addition, they are believed to contribute toward global warming and depletion of the ozone layer.

While the above four pollutants also originate from other sources, Environment Canada reports that a large share of each, and in some cases the greatest single share, is directly linked to the motor vehicles Canadians drive.

More vehicles, more drivers

Despite pollution problems associated with motor vehicle emissions, the number of vehicles on Canadian roads is growing. In 1989, there were 16.7 million registered vehicles on the road in Canada, 26% more than a decade earlier (13.3 million in 1979). Of these, 77% (12.8 million) were passenger automobiles in 1989.

While the number of Canadians with cars is increasing, the percentage of households owning a car has remained relatively stable since 1979. From 1979 to 1989, 77% to 80% of all households owned one or more automobiles, with an average of about 1.4 cars per household (among those households with a car). In 1989, nearly 2.4 million households, 25% of all households, owned two or more cars.

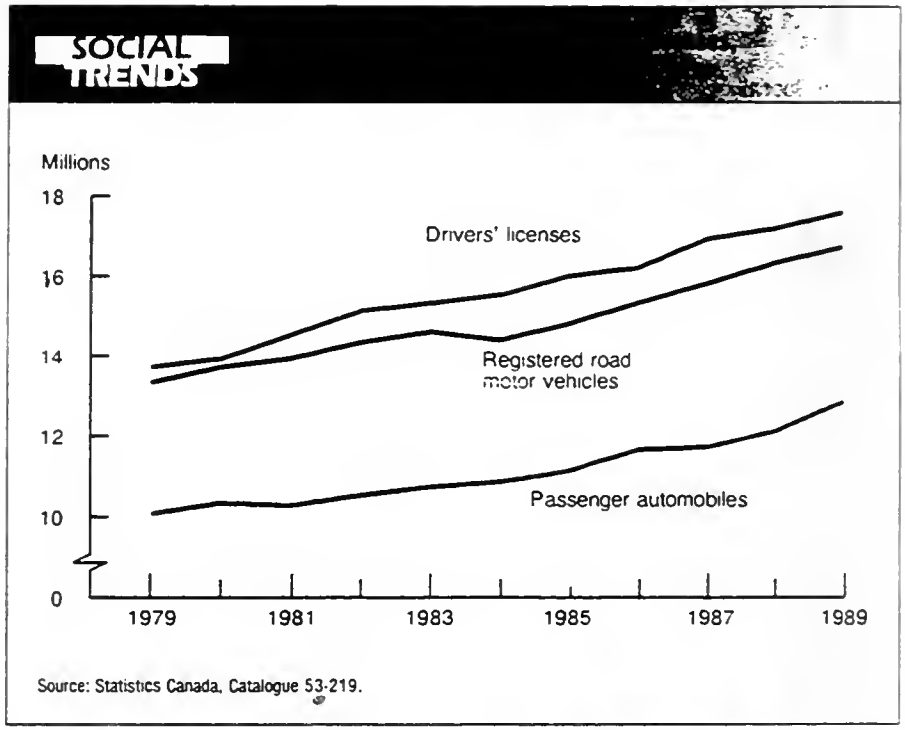
The percentage of people with a driver's license is also growing. The total number of Canadians licensed to drive increased to 67% of the population in 1989 (17.6 million people), from 58% (13.7 million) in 1979. Over the same period, the number of people of age to obtain a driver's license increased by 15% to 20.4 million. Further, in 1989, 86.3% of those old enough to drive were licensed to do so, up from 77.5% in 1979. These trends can be partially explained by the aging of the Canadian population.

More personal use cars driving further

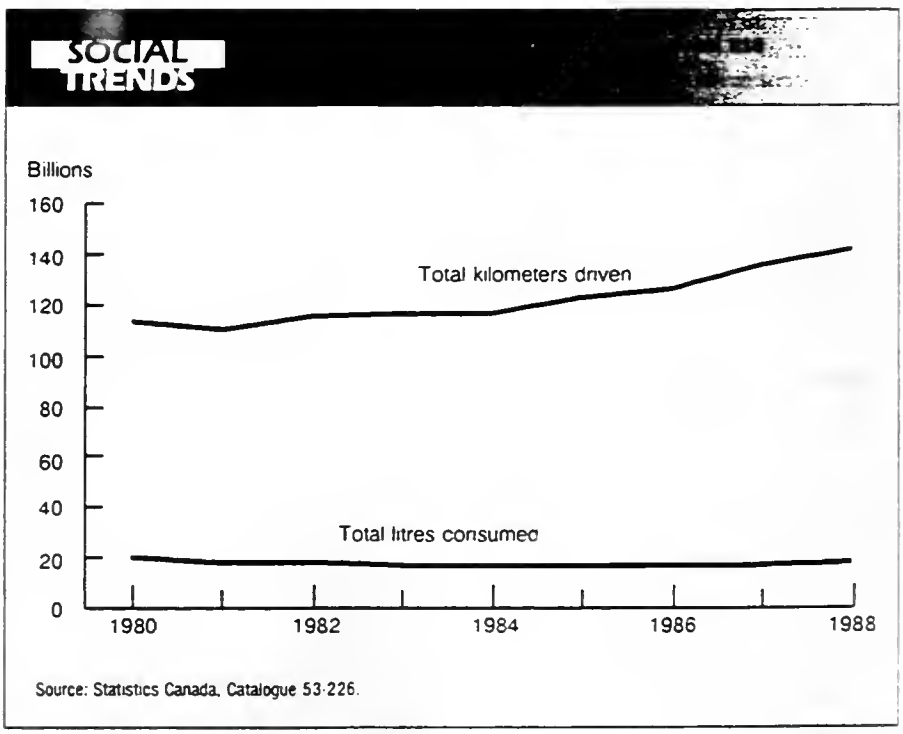
Between 1980 and 1988 (the years for which data are available), about one-half of all road vehicles — two-thirds of all cars — were personal use automobiles. These data include passenger automobiles licensed for the survey year, and exclude rental fleet operated, driver training, and antique vehicles. In 1988, an estimated 8.2 million cars were personal use vehicles, up from 7.1 million in 1980.

Personal use autos were estimated to have been driven a total of 141.8 billion kilometres (km) in 1988, compared with 112.9 billion in 1980. The average car was estimated to have been driven 17,380 km in 1988, the equivalent of 2.7 times from coast to coast — an average of almost 10% further than the 15,820 km driven in 1980.

Despite the increase in cars and the greater distances driven, personal use cars consumed less fuel in 1988 than they had in 1980. The average personal use car consumed about 2,090 litres of fuel in 1988, travelling approximately 8.3 km per litre burned. In comparison, during 1980, the average car consumed about 2,620 litres (6.0 km per litre). This resulted from the gradual replacement of heavier cars with large engines by lighter models with smaller engines and stricter emissions standards. In 1988, new 1987 model-year vehicles were estimated to have consumed, on



Source: Statistics Canada, Catalogue 53-219.



Source: Statistics Canada, Catalogue 53-226.

average, 26% less fuel than the new 1979 models had in 1980 — even though the new 1987 model-year cars were, on average, driven slightly (2%) further.

Public transit use also up

With more than three-quarters of the population living in urban areas (60% in Census Metropolitan Areas — CMAs), most Canadians have access to a mass transit system. Despite the increase in cars on the road, more Canadians are riding public transit than ever before. However, it is evident that most Canadians still tend to use personal rather than public transportation.

In 1988, urban transit systems[1] carried a total of 1.5 billion passengers, up 22% from 1.2 billion in 1978. Accompanying the increase in ridership has been a growth in the number of buses and other transit vehicles. The number of buses increased to 10,720 in 1988, up 17% from 1978. The total number of all transit vehicles increased about 14% over the same period, to 13,340 in 1988. They covered a total of 749 million km in 1988, up 20% from 1978. Still, this amounted to less than 0.01% of the total distance travelled by personal use cars in 1988.

Metropolitan Toronto peak-period transit usage patterns give some indication of the extent to which urban dwellers use personal rather than public transportation.

Toronto, Canada's most populous CMA (3.8 million people — 14% of all Canadians in 1990), is serviced by the country's largest urban transit system. According to the Metropolitan Toronto Planning Department, the number of people using peak-period (morning and afternoon rush-hour) transit services increased dramatically between 1975 and 1989. However, the proportion of commuters using these services increased only modestly. For example, from 1975 to 1989, the average total number of inbound person-trips crossing the central area (traffic monitor) cordon increased 21%, while the combined proportion of those using transit and commuter rail grew only 5 percentage points. Although the proportions of people using transit or commuter rail varied by the boundary crossed (outer metro, suburban, central and extended core boundaries), direction travelled, and time of day, the differences between 1975 and 1989 were generally modest.

At all monitored boundary crossings, the overwhelming majority of vehicles were cars. This is not surprising given that, in 1989, of the 1.34 million vehicles registered in Metro Toronto, 1.17 million (88%) were cars.

Most emission levels decreasing

Motor vehicles, especially those with poorly tuned engines, are major emitters of carbon monoxide (CO). Gasoline-powered cars and trucks were estimated to have produced about 54% of all carbon monoxide emitted in Canada in 1985. However, since the introduction of the catalytic converter in 1971, and the increased fuel efficiency of newer cars, urban area CO concentrations decreased 58% between 1974 and 1987. Toronto and Calgary reported the highest CO levels in 1986, although they exceeded the eight-hour maximum acceptable level less than 1% of the time.

In 1985, transportation accounted for nearly two-thirds of all nitrogen oxide emissions. Cars and other light-duty vehicles together emitted 24% of national

[1] Urban transit systems earning $250,000 or more per year.

Bus emissions

Environment Canada compared the emissions of a 1992 diesel transit bus capable of seating 43 people, and those of [illegible] cars built after 1988. [illegible] diesel bus, operating [illegible] produce emissions of approximately 54 times the carbon monoxide, 8.4 times the hydrocarbons and 2.4 times the nitrogen oxides produced by a bus carrying 43 people. Measured in total grams of pollutants emitted per mile, 43 cars would produce approximately eight to nine times the total emissions of one bus. The diesel bus, because of the type of fuel burned, would produce more particulate matter than the cars.

Environment Canada is currently examining alternative fuel and emission control systems that show potential to greatly reduce bus emissions.

SOCIAL TRENDS

Vehicles crossing central area cordon

1 Transit vehicles[1]
2 Trucks
3 Cars (includes taxis)

People[2] crossing central area cordon using:

4 Commuter rail
5 Cars (includes taxis)
6 Transit vehicles

[1] Excludes subway and commuter rail.
[2] Excludes taxi drivers.
Source: *Metro Cordon Count Program, 1989*, Metropolitan Toronto Planning Department, Transportation Division.

NO_X. However, despite a 45% increase in national NO_X emissions between 1970 and 1985, the annual average levels of nitrogen dioxide (NO_2) in urban centres have been consistently below the maximum desirable level since 1978.

Of the various oxides of nitrogen formed from motor vehicle emissions, NO_2 is the one of most concern to human health and the environment. While annual averages of NO_2 are within acceptable limits, and much lower than they once were, there are still occasions when those limits are exceeded. Between 1974 and 1987, average urban concentrations of NO_2 declined 29%. Decreasing NO_2 emissions from transportation accounted for much of the past reduction. However, because of anticipated increases in the number of vehicles on the road, it is expected that transportation will be largely responsible for a 6% increase in NO_X (and subsequent increase in NO_2) between 1985 and 2005.

In 1985, 40% of volatile organic compounds (VOC) were from transportation sources; cars alone emitted 22%. It is currently estimated that evaporative emissions from carburettors, gas tanks and losses during refuelling account for 40% of motor vehicle VOC emissions. The remaining 60% is from unburned gasoline released in exhaust, thus closely tied to poorly tuned engines.

Smog still a problem

During summer months, over one-half of all Canadians are exposed to ozone concentrations (smog) in excess of national air quality objectives. Most ground-level ozone resulting from human activity is generated within 100 km of densely populated areas and can extend as far as 200 km. The highest ozone concentrations usually occur during mid-afternoons within 50 km downwind of source areas.

The average urban concentration of ozone did not change between 1974 and 1987. However, the average number of hours of high ozone levels appears to have increased between the end of 1985 and 1989. Between 1983 and 1989, Saskatoon and Victoria were the only major Canadian urban areas (population of 50,000 or greater), where routine monitoring takes place, that did not record ozone levels in excess of maximum acceptable concentrations. Ozone levels in the regions of the British Columbia Lower Mainland, the Windsor-Quebec City corridor, and those of Saint John, New Brunswick and western Nova Scotia, most frequently exceeded environmental quality requirements. Maximum acceptable limits were exceeded

Pollution monitored

In an effort to better monitor and regulate air pollution and pollution control programs in Canada, the federal and provincial governments jointly established the National Air Pollution Surveillance (NAPS) network. As of 1989, the network included 380 monitors at 132 stations and covered 52 Canadian urban areas.

Since the 1970s the NAPS network has been providing information on common air pollutants in Canada. Some of the pollutants monitored include particulate matter, sulphur dioxide, carbon monoxide (CO), nitrogen oxides (NO_X), hydrocarbons in general and volatile organic compounds (VOC) in particular, and ground-level ozone (formed from the interaction of NO_X and VOC in sunlight).

Based on measurements taken by the NAPS network, a combined total of more than 22.2 million tonnes of the above pollutants were estimated to have been emitted into the air in 1985. At least 30% of the total tonnes emitted were estimated to have originated from gasoline powered cars and trucks. Further, as measured by many NAPS monitors, daily peak levels of some emissions coincide with rush-hour traffic flows.

However, because of variability in the state of vehicle emission control systems, engine efficiency and differences in personal driving habits, motor vehicle emissions could actually be much higher than are currently estimated. For instance, new cars built between 1975 and 1987 emitted 4.9 times the hydrocarbons, 7.4 times the CO and 3.1 times the NO_X emitted by new cars built since 1988. Passenger cars built before the introduction of emission controls produced more than 21 times the hydrocarbons, 25 times the CO, and 3.6 times the NO_X emitted by 1988 and newer models. Older vehicles in poor mechanical condition, with emission control devices rendered inoperative by owner ignorance or intent, could result in emissions as much as 50 times higher than those of newer vehicles.

Although individual vehicle emissions have been decreasing since the introduction of emissions regulation in 1971, it is expected that current emission levels are, at best, temporarily stable at or near 1985 levels. Anticipated increases in the number of motor vehicles are expected to contribute toward emission increases unless stricter emission controls are adopted

within the Windsor-Quebec City corridor (home to most Canadians) more often than in any other Canadian region. In 1988, one hour concentrations exceeding acceptable limits were recorded 189 times in Windsor, 157 in a Toronto suburb (North York), 149 in London, and 122 in Oakville.

During high concentrations, those most at risk include people with asthma or lung disease, children, the elderly, and individuals who work or exercise heavily outdoors. While other emission products also harm vegetation, ground-level ozone in particular is known to adversely affect crops, trees, vegetables, and ornamental plants. When average ozone concentrations ranged from 40 to 50 parts per billion (levels below the maximum acceptable limits), annual yield losses to selected Ontario crops were recently estimated to range from 1% to 12%. Depending on the frequency of high ozone episodes, the combined commercial losses can range from $17-$70 million per year. Crops in the Lower Fraser Valley area of British Columbia are believed to suffer similar damage.

Reducing emissions

Acting on recommendations made by participants in Canada's Green Plan Consultations, the Canadian Council of Ministers of the Environment (CCME) have developed a 10-year national plan to manage emissions of nitrogen oxides and volatile organic compounds. Through this plan, federal and provincial governments hope to co-operatively resolve problems of ground-level ozone by the year 2005.

One of the key elements of the plan is to reduce the regional levels of motor vehicle NO_X and VOC emissions. There are a variety of strategies suggested to achieve this goal. They include public education campaigns to encourage energy conserving driving habits and alternative modes of transportation, more effective speed limit enforcement, ozone management plans for urban centres of 100,000 or more people, the adoption of new and stiffer emission standards by 1996, and mandatory motor vehicle inspection and maintenance programs in the Lower Fraser Valley and Windsor-Quebec City corridor regions by 1993. These and other strategies are forecasted to reduce the total on-road vehicle NO_X emissions by 32% and VOC emissions by 35% by the year 2005.

The cost of the first phase of the plan is estimated at more than $850 million (1989 dollars), half of which is related to mobile sources (primarily cars and light-duty trucks). It is expected that vehicle owners and operators will ultimately pay the costs associated with reducing vehicle and fuel emissions. However, in the absence of good alternative modes of transportation, the plan may limit people with low incomes from access to employment and other opportunities.

To date, Ontario, Quebec and Newfoundland are the only provinces to prohibit tampering with emission controls. However, in the spring of 1992, British Columbia plans to launch a comprehensive and compulsory vehicle emissions testing program.

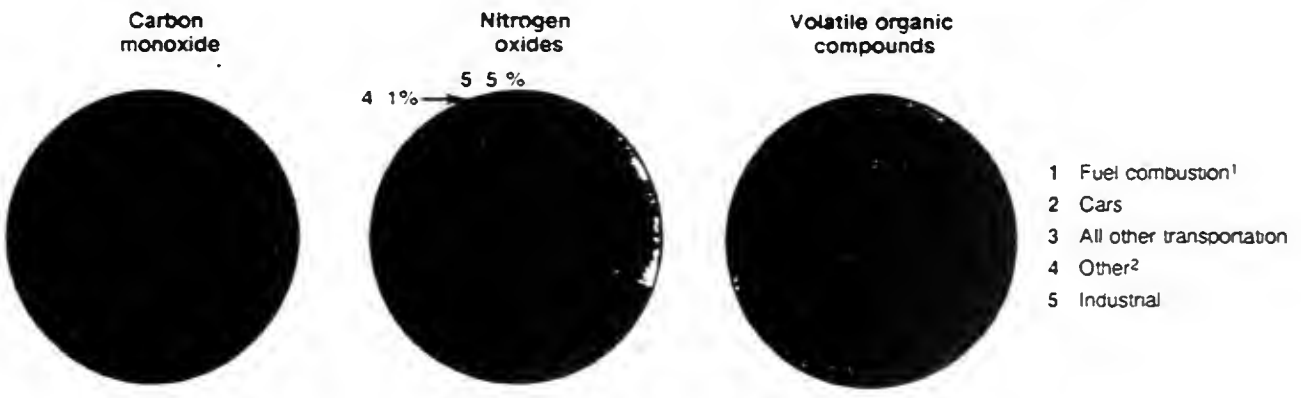
The Aircare program

British Columbia is launching Canada's first mandatory motor vehicle emissions testing program in the Spring of 1992. At an estimated cost of $40 million, the program (known as Aircare) will require that most cars, motorhomes, and light-duty trucks registered in the British Columbia Lower Mainland pass annual emission inspection prior to renewal of the vehicle's licence. Heavy duty vehicles will come under a commercial vehicle inspection program. The program may be expanded to other areas of the province in the future.

The Aircare program was developed in response to studies by the Greater Vancouver Regional District that established that motor vehicle emissions were the main cause of air pollution in the Lower Mainland of British Columbia. Motor vehicles were responsible for 53% of the hydrocarbons, 64% of the nitrogen oxides and 90% of the carbon monoxide in the Lower Mainland. Because of those emissions, the region (home to more than 50% of British Columbia's population) has one of the highest levels of ground-level ozone in the country.

Testing by B.C. Environment and Environment Canada found that 54% to 70% of vehicles currently on the road have emissions-related defects. It is anticipated that most vehicles tested will need repairs and that 25% to 30% of vehicles will actually fail emission testing. The most commonly required repair to reduce emissions to acceptable limits is a tune-up.

SOCIAL TRENDS



[1] Refineries, commercial and residential, power plants, and other.
[2] Incineration, fuel marketing, structural fires and slash burning, solvent use, and other.
Source: Kostaltz, Anthony M., *Canadian Emissions Inventory of Common Air Contaminants (1985)*, Environment Canada.

The City of Calgary Transportation Department has also initiated a program that is the first of its kind in Canada. Effective May 31, 1991, Calgary launched its Air Improvement Resolution (AIR) program to create awareness of air pollution originating from motor vehicle emissions and alternative modes of transportation. Under the AIR program (modelled after two successful programs in the United States), commuters are encouraged to find alternatives to driving alone in their car two days per month.

Definitions

Carbon monoxide (CO) is a colourless toxic gas that causes deaths associated with exhaust entering the passenger compartment of a vehicle. CO is also believed to convert to carbon dioxide (CO_2), a major "greenhouse gas," within a few months of emission into the atmosphere. In 1987, it was estimated that cars produced more than 10% of national CO_2, thereby contributing toward global warming.

Nitrogen oxides (NO_x) are a group of gases composed of only nitrogen and oxygen. As a group, oxides of nitrogen may remain airborne for several days, travel thousands of miles, and form acid rain, ground-level ozone, and other environmentally or human health damaging products.

Volatile organic compounds (VOC) are a sub-group of hydrocarbons. Although many VOCs are also produced naturally, those generated by human activity have greater potential to contribute to ground-level ozone formation and other serious human health and environmental problems.

Ground-level ozone (smog) is formed in a complex series of reactions between NO_x and VOC, using energy provided by solar ultraviolet radiation. Although it is chemically the same as the stratospheric ozone (commonly known as the ozone layer — the layer that filters out ultraviolet light before it reaches earth), the effects of ground-level ozone on plant, animal, and human health are negative. It is the major component of urban smog.

Changing fuel an alternative

Environment Canada and other agencies have been studying the emissions levels associated with burning methanol, ethanol, propane, and natural gas. To date, natural gas appears to offer the greatest potential as a gasoline substitute. However, as with gasoline-fuelled vehicles, potential emission reductions are maximized only if the latest technologies are employed and vehicles are kept well tuned. Further, the cost of adapting the gasoline distribution system to accommodate new fuels is nearly prohibitive — a cost that would almost certainly be passed on to the consumer.

Changes to gasoline have also reduced emissions associated with its use. For example, its ozone forming potential has been reduced through changing its volatility (evaporative properties). Additional reductions of volatility are planned for the future. As well, the Canadian ban on the sale of leaded gasoline has largely eliminated particulate lead from the air. Following regulations limiting the lead content of gasoline and the increased use of vehicles designed for unleaded fuel, ambient lead levels dropped 86% between 1973 and 1987. With leaded gasoline now unavailable to the general public, it is likely that lead emission levels have now decreased to nearly zero.

Changing driving and vehicle maintenance habits may result in notable emission reductions, regardless of the type of fuel used. Driving at 90 km/hr rather than 100 km/hr may reduce fuel consumption by as much as 10% and lower emissions. Stricter enforcement of existing speed limits may provide the incentive to drive more slowly. Keeping vehicle engines well tuned may also cut fuel consumption by as much as 10%.

Conclusion

The benefits of increased use of mass transit and other alternatives to personal vehicles cannot be accurately forecasted. However, in a general sense, decreasing the use of personal vehicles, driving them more slowly and maintaining them so that they meet model-year emission standards, will certainly contribute toward improved air quality. A decrease in the number of vehicles on the road should also reduce traffic congestion, parking problems, and the level of national energy consumption.

While alternatives to personal motor vehicle transportation are not yet used by the majority of Canadians, there are indications that public transit is used more often now than a decade ago. Current plans to stiffen vehicle emission standards and implement compulsory emission testing, combined with the rising costs of vehicle ownership and operation, and heightened public concern about environmental issues, may help to reduce dependence on personal vehicles.

References

The emissions information contained within this article has been drawn from a number of Environment Canada sources. Additional information concerning the sources, quantities and effects of the emissions discussed in this report may be obtained from Environment Canada or by referring to the following government publications:

1) Hilborn, J. and M. Still, *Canadian Perspectives on Air Pollution*, Environment Canada.

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4) *Management Plan for Nitrogen Oxides (NO_x) and Volatile Organic Compounds (VOC): Phase I. November 1990*, Canadian Council of Ministers of the Environment.

5) *National Urban Air Quality Trends 1978-1987*, Environment Canada

Alan Goodall *is an analyst with* Canadian Social Trends.

APPENIDX A-3 "Bioconversion of Waste Paper to Ethanol"

Process Biochemistry 27 (1992) 239–245

Bioconversion of Waste Paper to Ethanol

Morris Wayman, Shu Chen & Kim Doan

Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Canada M5S 1A4

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A preferred process is described for efficient bioconversion of waste paper to ethanol. The paper was given a short saccharification by cellulase enzymes at 45 °C followed by fermentation in the continued presence of the enzymes at 37 °C. Ethanol yields were 350–400 litres/t of waste paper, at 8% ethanol by volume. In some of these runs, commercial cellulase was used, in others our laboratory preparation of cellulase was used; in both cases a small amount of cellobiase was also needed. There was no pretreatment before saccharification as is common with lignocellulosics. A new fermenter design well suited for waste paper processing or for cellulase production is described. The amount of paper in municipal solid waste in North America is about 100 million t yearly, enough, upon bioconversion, to replace 16% of all North American gasoline by ethanol.

INTRODUCTION

Uncertainty about the supply of crude oil from the Middle East, the associated high cost of maintaining that supply, and concerns about atmospheric pollution attributed to the use of petroleum-based motor fuels has re-awakened technical interest in cellulosics as a source of ethanol as motor fuel. Recent research has been reviewed by Wayman & Parekh.[1] The vast quantity of household trash (municipal solid waste, MSW) generated annually in the advanced industrial countries is a further stimulus for such work. The amount of waste paper generated annually in North America and finding its way to landfill sites as MSW is about 100 million t, enough, upon bioconversion, to replace about 16% of all North American gasoline by ethanol.

The prospect of conversion of so much MSW paper to fuel ethanol has resulted in considerable pilot plant and experimental activity. In 1976, a facility to convert the cellulose content of MSW to ethanol was installed at the Gulf Chemicals Pittsburgh, Kansas, petrochemical complex. It had a nominal capacity of 1 t of feedstock per day, enough to produce 80000 litres of ethanol yearly. The process employed cellulase enzymes produced on site by the fungus *Trichoderma reesei*, saccharification being followed by fermentation by common bakers' yeast *Saccharomyces cerevisiae*. In 1980 the feedstock was changed to steam-exploded aspen wood, based on the prospect of dedicated planta-

Corresponding author: Dr M. Wayman. Telephone: (416) 9784905; Fax: (416) 9788605

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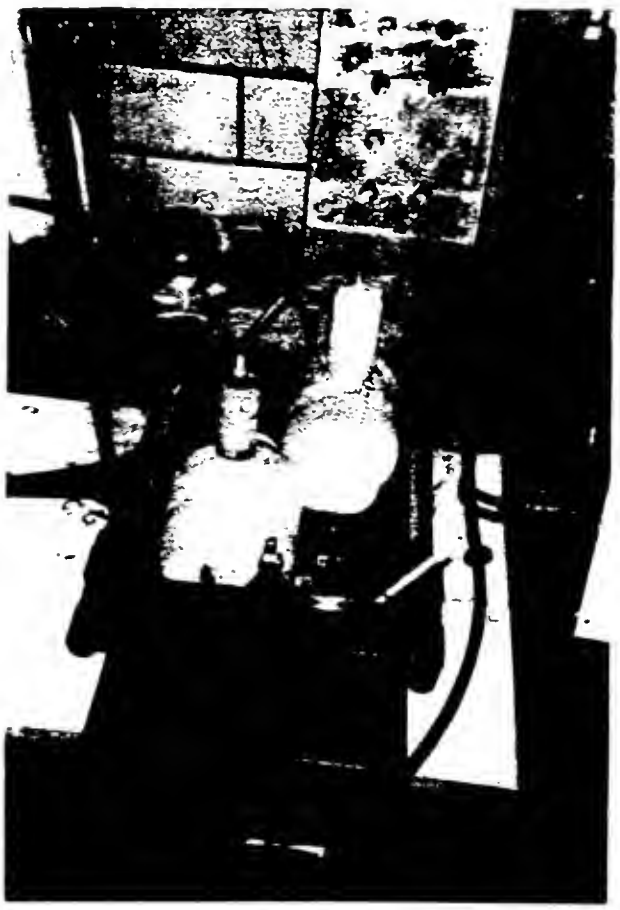

Fig. 1. 2·5 litre reciprocal shaking bioreactors.

tions. The operation, which was discontinued for lack of funding in 1982, has been described by Emert & Katzen.[2]

In 1985, Procter and Gamble Paper Products sulphite pulp-paper mill in Mehoopany, Pennsylvania, faced a problem with excessive fibres in their effluents, and established a pilot plant for bioconversion of the cellulosic fibres to ethanol, at the same time considering MSW as a possible feedstock (Bob Barkley, Procter and Gamble, private communication). The pilot plant took in 3–5 t of fibres each day. There was no pretreatment before saccharification, since the cellulose had already had extensive 'pretreatment' in the pulping. Simultaneous enzymatic saccharification and fermentation was practised, using purchased cellulase enzymes.[3] After about 1 year of operation, the project was abandoned; contributing factors to the pilot plant closure being the high cost of purchased enzymes, and the expected high capital cost for a complete cellulosic ethanol plant.

A pilot plant for the bioconversion of lignocellulosics to ethanol was built by Institut Français du Petrole at Soustons, France, using Stake steam pretreatment,[1] followed by saccharification by cellulase enzymes made on site, followed by fermentation.[4] This pilot plant can process 3 t/h of cellulosics, including, presumably, MSW.

The Tennessee Valley Authority (TVA) has long been associated with bioconversion of wood to ethanol,[1] and has recently studied bioconversion of MSW, using dilute acid pretreatment and acid hydrolysis followed by fermentation.[5]

The diversity of pilot plant designs illustrates that no single process for bioconversion of cellulosics or MSW paper has yet emerged as best. There are several technical questions which remain to be answered: is pretreatment of paper before saccharification necessary? Is enzymatic saccharification to be preferred to acid hydrolysis? Can enzymes be made cheaply on site? Should saccharification be complete before fermentation, or should the two process steps be simultaneous? How can the difference between optimal temperature of saccharification (45–50 °C) and fermentation (30–32 °C) be reconciled? Should the waste paper be added all at once at the beginning, or is fed batch more productive? This paper will attempt to answer these questions. To assist assessment of various options, performance targets were established: 350–400 litres ethanol per tonne of waste paper: ethanol concentration of 6+% by volume; and 54 h maximum total time for saccharification and fermentation.

MATERIALS AND METHODS

Materials and microorganism

A variety of waste paper samples were collected locally. The commercial cellulase used in this work was Multifect S-850 (Finnish Sugar Company, Finland, 344 IU/g,[6] 37·9% protein). Also, cellulase produced in our laboratory[7] was used. The cellobiase was Novozym 188 (Novo, Denmark, 91·5 IU/ml). Fermentation was by *S. cerevisiae*, Fleischmann's yeast purchased in a local grocery store.

Analytical procedures

Paper samples were analysed by dissolving the cellulose in 72% sulphuric acid at room temperature, diluting with cooling to 4% sulphuric acid, heating in an autoclave at 121 °C for 1 h, filtration of the lignin, neutralization with lime and, after vacuum evaporation, analysis of the resulting sugar solution by HPLC (Sugar Analyser 1, Waters,

Table 1. Waste Papers. Summative Analysis and Ethanol Potential[a]

	Shredded white office paper	*Newspaper*	*Kraft paper*	*Milk carton*	*Corrugated carton*	*Paperboard*	*Mixed waste paper*[b]
Moisture (%)	6·6	8·9	5·5	5·9	6·0	6·8	6·3
Ash (%)	9·1	0·3	1·3	0·1	1·1		2·8
Carbohydrates as sugars (%)	88·0	70·9	81·3	77·2	69·1	77·6	78·3
Ethanol potential (litres t)	568	457	524	498	446	500	508

$$\text{Ethanol potential} = \frac{\text{carbohydrates as sugars} \times 0{\cdot}51 \times 10}{0{\cdot}79} \text{ litres t}$$ Cellulose ethanol potential = 710 litres/t.

[b] Mixed waste paper used here is 25% white office paper, 25% newspaper, 20% kraft paper, 20% milk carton, 10% corrugated carton. Ethanol potential is calculated from this composition.

with an Aminex HPX 87P column using water at 65 °C as the eluant, a refractive index detector and 1·6% *n*-propanol as internal standard). Ethanol was determined by GC (Hewlett Packard 5700A, with an ionization flame detector, using 0·7% *n*-propanol as internal standard).

Saccharification and fermentation

None of these paper samples were given any pretreatment before saccharification. The paper was shredded and cut into squares 1–2 cm.

Saccharification was carried out in 250 ml shake flasks containing 100 ml saccharification medium, or in 2·5 litre fermenters designed for the processing of paper, as described below. The amount of paper was initially 2–20% solids. The amount of cellulase enzymes used was 8·6–70 IU/g paper, the enzyme being a mixture of 70% cellulase and 30% cellobiase. The pH was adjusted with lime to 4·8. Small amounts of Vitamin B_{12} and trace elements were added, along with a few drops of Triton X100. Saccharification in the 250 ml shake flasks was assisted by several small stainless steel balls. Incubation was at 45 °C in a rotary shaker at 150 rev/min.

Fermentation was begun either when saccharification had proceeded for 6 h, or after it was apparently complete. In either case, the temperature was reduced to 37 °C and the yeast added. This temperature was chosen to permit saccharification to continue during fermentation. Nutrients added for fermentation were either YMP (0·3% yeast extract, 0·3% malt extract, 0·5% peptone) or DAP (0·01% diammonium phosphate, 0·02% urea). Initial cell levels were 10–20 g/litre freeze dried weight. Anaerobic closures were used.

For larger scale saccharification and fermentation, conventional stirred tank fermenters proved unsatisfactory, since the paper clogged the internals even at low consistency. A simple, efficient bioreactor designed for paper saccharification and fermentation is shown in Fig. 1. The body of the bioreactor is a (recycled) plastic 2·5 litre chemical bottle, held horizontally in a reciprocal shaker water bath with temperature control. A 100 ml bottle is sealed to a hole in its side, the bioreactor being oriented so that this smaller bottle is vertical, serving to keep the gas outlet at its head free of paper. The gas outlet is equipped with an anaerobic closure. In operation, the bioreactor cycles about 120 times per minute, enough to keep the paper in suspension even at high consistency. For use in aerobic fermentations, such as for enzyme production, an air inlet and air flowmeter were added. Usually in this bioreactor 100 g of waste paper was processed suspended in 1 litre of medium, and after partial liquefaction a second 100 g of waste paper was added.

RESULTS AND DISCUSSION

Composition of waste papers

Table 1 presents the results of analysis of various waste papers. The carbohydrate content expressed as monomeric sugars ranged from 69·1% in corrugated paper to 88·0% in shredded white office paper (SWOP). Ash content was very low in milk carton and newspaper and high in office paper. The potential for ethanol production ranged from 446 litres/t for corrugated carton to 568 litres/t for SWOP. Pure cellulose upon saccharification and

Table 2. Saccharification of Shredded White Office Paper (SWOP). 100 ml Saccharification Medium in 250 ml Shake Flasks

E SWOP (IU/g)	Solids (%)	(h)	Total sugars (% of SWOP)	Saccharification (%)
8·6	5	48	57·2	65·0
		72	61·4	69·8
17·2	10	48	70·8	80·5
17·2	[b]10 + 10	72	47·6	54·1
34·4	10 + 10	72	43·6	49·5
34·4	10 + 10	72	48·8	55·5

[a] E is 70% cellulase (Multifect S-850), 30% cellobiase (Novozym 188).
[b] 10 + 10 represents 2 additions of SWOP 24 h apart.

Table 3. Simultaneous Saccharification and Fermentation of Shredded White Office Paper (SWOP)

E SWOP (IU/g)	(h)	Ethanol			
		g/litre	% by vol	litre/t	g/g glucose[b]
17·2	72	55·8	7·1	353	0·41
17·2	96	59·3	7·5	375	0·44
34·4	72	63·0	8·0	395	0·47
34·4	96	65·8	8·3	415	0·49

[a] SWOP composition, in %: moisture 1·06, acid insolubles 10·0, glucose 65·8, xylose 13·6, mannose + arabinose 3·8.
[b] The figures in the last column assume that all of the glucose and one-half the (mannose plus arabinose) are available for fermentation.

fermentation can theoretically yield 710 litres ethanol/t.

Ethanol from waste paper

Table 2 reports the results of saccharification of shredded white office paper (SWOP) by a 70:30 mixture of cellulase and cellobiase in 100 ml saccharification medium in 250 ml shake flasks at pH 4·8, incubated at 45 °C on a rotary shaker at 150 rev/min with the additives named in the section 'Saccharification and Fermentation' above. With 8·6 IU enzyme/g paper at 5% solids over a period of 72 h, 69·8% saccharification was obtained. When the ratio of enzyme to paper and the percentage of solids were both doubled, yields of sugars were 80·5% in 48 h.

Simultaneous saccharification and fermentation of SWOP gave the results shown in Table 3. This is not a truly *simultaneous* saccharification-fermentation: SWOP was treated with saccharification medium at 45 °C for 6 h, then cooled to 37 °C and yeast and YMP added. In this procedure, to 100 ml enzyme solution containing 240 IU cellulase and 27 IU cellobiase plus the additives listed above, 10 g SWOP was added, incubated on a rotary shaker at 45 °C for 6 h, then cooled to 37 °C, and yeast and nutrients were added for anaerobic fermentation. After 24 h, the same amount of SWOP and enzymes were added and the mixture incubated for an additional 48 h. Ethanol was produced at the rate of 353–415 litres/t SWOP, at concentrations from 7·1 to 8·3% ethanol by volume. Comparing the results of saccharification shown in Table 2 with those of simultaneous saccharification and fermentation shown in Table 3 it is apparent that saccharification proceeds more efficiently in the presence of yeast.

The results shown in Table 3 meet two of the targets set forth in the introduction: the ethanol yields are above 350 litres/t and the ethanol concentration is above 6% by volume. However, both the total processing time and the amount of enzyme acquired exceed our targets. Enzymes are now made from waste newspaper[7] or other inexpensive inducers,[8] so the amount of enzyme used may be tolerable. Processing time may be improved by adaptation of the yeast to this medium.[10]

Simultaneous saccharification-fermentation of mixed waste paper resulted in 306 litre ethanol/t at a concentration of 6·1% ethanol by volume in 72 h, about 87% of the value obtained with SWOP. Waste paperboard packaging yielded ethanol at the rate of 347 litres/t, at 1·7% ethanol by volume in a low density run.

Bioconversion process variations

Several variations on the above saccharification-fermentation procedure were investigated, with the objective of meeting all the targets set out in the Introduction.

Variation 1

SWOP (5 g) was added to 100 ml solution containing 43 IU mixed enzymes and incubated at 45 °C, and after 24 h incubation the sugar solution was removed by centrifugation. An additional 43 IU mixed enzymes was added to the residual paper, and after another 24 h incubation at 45 °C the new sugar solution was separated by centrifugation. The two sugar solutions were combined for analysis. Saccharification by this fed batch enzyme addition was complete, and fermentation of the combined sugar solution was very rapid, being complete in 6 h, to yield 438 litre ethanol/t, the best of any of the

procedures, but at very low ethanol concentration, 1·1% by volume.

Variation 2

In an attempt to raise ethanol concentration, a run similar to Variation 1 was made beginning with 10 g SWOP in 100 ml enzyme solution containing 344 IU enzymes, and incubated at 45 °C for 24 h. Then the same amount of enzyme was added two more times at 24-h intervals.[4] Ethanol yield at 24 h was 411 litre/t, at 4·0% ethanol by volume.

Variation 3

In this procedure, 20% SWOP solids was obtained by adding the paper and enzymes in two equal portions with a 6-h interval, for a total of 1376 IU enzyme. Fermentation was slow, ethanol yields being 336 litre/t at 96 h, and ethanol concentration reaching 6·7% by volume.

Variation 4

This is low solids, fed batch SWOP, 2 g SWOP plus 138 IU enzyme being added 10 times over 5 days. After 24 h fermentation, ethanol yield was 375 litres/t, at 6·8% ethanol by volume.

None of these variations met all the performance targets. The results shown in Table 3 are the best so far in meeting these targets.

Bioconversion with laboratory-prepared cellulase

Enzyme solution was prepared according to the procedure of Chen & Wayman[7] in the 2·5 litre fermenter (Fig. 1) modified to provide a slow air flow over the fungal fermentation. The solution contained 1480 IU and 1·92 g protein/litre. To 100 ml enzyme preparation (a) after filtration or (b) without filtration, was added 5 g SWOP + 9·15 IU cellobiase. After adjustment of pH to 4·8 and incubation at 45 °C for 6 h, the suspension was cooled to 37 °C and 1 g bakers' yeast + YMP was added. Anaerobic fermentation yielded ethanol at the rates of (a) 292 and 359 litres/t SWOP in 24 and 48 h, and (b) 319 and 400 litres/t SWOP in the same periods. The laboratory-prepared enzyme is therefore effective,[5] but there remains a requirement for cellobiase. It is more effective when not filtered, but used as the entire enzyme production mix, which may save added nutrients.

Bioconversion in the 2·5 litre fermenter

In an initial run with this fermenter, commercial enzyme was added as a fed batch. To 1000 ml enzyme solution containing 2408 IU cellulase and 275 IU cellobiase and the usual additives was added 100 g SWOP, the pH adjusted to 4·8 with lime, and the mixture incubated at 45 °C. At 24 and 48 h, fresh enzyme was added in the same amounts. After a total of 96 h at 45 °C and a total of 8049 IU enzyme, the mix was cooled to 37 °C, 20 g yeast and YMP were added. During 6 and 24 h of fermentation, ethanol was produced at the rate of 324 and 391 litres/t SWOP, and 3·2 and 3·9% ethanol by volume. Samples taken at 72 and 96 h of saccharification showed no increase of sugar production, in both cases about 71% of theory, suggesting that fermentation could have begun 24 h earlier.

Another run was made in which the 2·5 litre fermenter was used for both cellulase production[7] and for fermentation. The enzyme solution so made contained 1480 IU and 1·92 g protein/litre. To 1 litre of this enzyme solution was added 91·5 IU cellobiase and 50 g SWOP, plus a few drops of Triton X100, the pH was adjusted to 4·8 and the suspension was agitated in the reciprocal shaker at 45 °C for 6 h. It was then cooled to 37 °C and yeast and YMP were added. After 24 h fermentation, ethanol yield was 340 litres/t SWOP, at 1·7% ethanol by volume. At this time, an additional 50 g SWOP was added plus an additional 91·5 IU cellobiase, and agitation was continued for another 24 h, resulting in an ethanol yield of 320 litres/t SWOP at an ethanol concentration of 3·2% by volume. These yields, while somewhat lower than obtained in the initial run, are good considering that much less cellulase was used (1480 IU compared to 7224 IU) and less cellobiase (183 IU compared to 825 IU); the usual Vitamin B_{12} and trace elements were not added; and the total time was much shorter (54 h compared to 78 or 120 h). Cellulase usage of 14·8 IU/g waste paper is acceptable.

CONCLUSIONS

1. The questions asked in the last paragraph of the Introduction can now be answered, at least in part. Pretreatment before saccharification is not necessary for efficient bioconversion to ethanol of the papers we have studied. Enzymatic saccharification is to be preferred to acid hydrolysis because the yields are much higher and the conditions gentler. Cellulase enzymes can be made cheaply on site. The best relationship between time devoted to saccharification and that to fermentation in a modified

semi-simultaneous saccharification fermentation is still a study objective: however, the process proposed in the next paragraph is satisfactory. In our practice, saccharification at 45 °C combined with fermentation at 37 °C has permitted saccharification to continue at an acceptable rate during fermentation. In Ref. 3, fermentation was carried out at 40 °C combined with stripping of the ethanol as produced. This procedure merits further exploration. Fed batch of paper, and perhaps of enzyme, are better than when these are added all at once, in part because paper solids above 10% are difficult to keep in suspension but are necessary for high ethanol concentration, however after partial saccharification the mix becomes very fluid and more paper can be added. Saccharification is more efficient at low solids, which combined with a fed batch mode of operation can produce high ethanol concentration.

2. A 'best' process for bioconversion of waste paper can be specified as follows: a saccharification medium containing enzymes plus additives (Vitamin B_{12}, trace elements, Triton X100) is prepared. The enzymes are a mixture of cellulase and cellobiase in the ratio of 70:30 (or 80:20[3]), the amount being about 34 IU/g of the initial paper addition. The pH is adjusted to 4·8 with lime, and waste paper divided into reasonably small pieces, about 1–2 cm^2, is added to a density of 8–10%. This is incubated with agitation at 45 °C for 6 h. The temperature is then reduced to 37 °C and yeast and YMP added, the yeast at 10–20 g/litre, the YMP at 3, 3 and 5 g/litre. Agitation is continued for 24 h when a second batch of paper and enzyme is added. Incubation and agitation are continued for 24–48 h. The ethanol so produced should be at the rate of 350+ litres/t waste paper at a concentration of 6% by volume or more.

3. A new, inexpensive benchtop fermenter suitable for waste paper processing or for cellulase production is described.

4. Cellobiase is added to saccharification to hydrolyse cellobiose, a product of cellulose hydrolysis and an inhibitor of it. The use of cellobiose-fermenting yeasts[11] should remove the need for cellobiase addition. Some of these yeasts also ferment xylose, an added advantage when hardwood-containing papers are being processed.

5. The potential for ethanol production from MSW is about 200 litres/t. The amount of MSW in North America is about 260 million t each year, sufficient for bioconversion to 52000 million litres annually (13000 million US gallons each year). This amounts to 16% of all motor fuel used in North America. The USA now imports nearly half of its crude oil, total oil consumption being about 1 million million litres/year (17 million barrels/day; 1 barrel equals 160 litres), compared to domestic annual production of 540000 million litres, leaving a gap of 460000 million litres of crude oil to be filled by imports. Upon refining, the amount of motor fuel produced is less than half the crude oil volume, such that 1 litre of motor fuel comes from 2·2 litres of crude oil. Thus 1 litre of fuel ethanol replaces 2·2 litres of crude oil. The potential production of ethanol from MSW can therefore displace 114000 million litres of crude oil, about 25% of imports, the amount normally imported from the Middle East. The potential production of ethanol from MSW is large enough to have significant political impact. Similar calculations can be made for Europe.

ACKNOWLEDGEMENTS

This research is supported by the Ontario Ministry of the Environment and by the Natural Sciences and Engineering Research Council of Canada.

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APPENDIX A-4 "Pretreatment of Waste Paper in a Vapour Cracker and Bioconversion of the Pretreated Fibre to Ethanol"

Pretreatment of waste paper in a vapour-cracker and bioconversion of the pretreated fiber to ethanol

E. Capek-Ménard, P. Jollez and E. Chornet

Département de génie chimique, Université de Sherbrooke
Sherbrooke (Québec), Canada J1K 2R1
Phone: (819) 821-7171; Fax: (819) 821-7955

M. Wayman and K. Doan

Department of Chemical Engineering and Applied Chemistry
University of Toronto, Toronto, Ontario, Canada M5S 1A4
Phone: (416) 978-4905; Fax: (416) 978-8605

Abstract

The vapour-cracker, a variation of the generic technology known as "steam explosion guns" has been used for the pretreatment of waste paper for bioconversion to ethanol. Paper fibres were soaked in 0.5% sulphuric acid under vacuum and heated in the steam vapour-cracker for 2 minutes, followed by enzymatic saccharification and fermentation. Control yields (no pretreatment) were at the rate of 357 liters per tonne of waste paper, while after the sulphuric acid pretreatment ethanol yields of 460 liters per tonne of waste paper were 30% over controls and 91% of theoretical. Most benefit was gained in the first 24 hours of fermentation.

Sommaire

Le vapo-craqueur, une variante de la technologie générique appelée communément "explosion à la vapeur" a été utilisé comme prétraitement de papiers usés avant leur bioconversion en éthanol. Les papiers ont été imprégnés sous vide avec une solution ayant 0.5% (pondéral) acide sulfurique et prétraités à différentes températures pendant 2 min. Le prétraitement a été suivi d'une hydrolyse enzymatique et de fermentation. Les rendements, sur le papier non-prétraité, ont donné 357 litres d'éthanol par tonne de papier usé alors que le prétraitement à l'acide sulfurique résulte en 460 litres d'éthanol par tonne de papier usé, c'est-à-dire 30% de plus que pour le papier non-prétraité et 91% du rendement théorique. La plupart de l'augmentation en rendement est atteinte dans les premières 24 h de la fermentation.

Keywords: vapour-cracker, pretreatment, waste paper, saccharification-fermentation, ethanol

Introduction

Conventional processes for bioconversion of lignocellulosic biomass to ethanol or other useful fuels or chemicals include the steps of reducing the feedstock to a convenient particle size, pretreatment with steam or dilute acid at elevated temperatures for a short time, followed by enzymatic saccharification or acid hydrolysis, then by fermentation of the resulting sugars to ethanol or other desired products. This sequence is subject to several variations (Wayman and Parekh, 1990, especially Chapter 16). The present paper describes novel equipment for pretreatment. Pretreatment of lignocellulosics performs several valuable functions: it hydrolyses the hemicellulose largely or entirely to monomeric sugars such as glucose, xylose, arabinose, mannose and galactose, or to low oligomers of these. In so doing, it sets free the lignin, rendering it soluble in dilute sodium hydroxide for recovery if desired; and the insoluble cellulosic residue is expanded to a lacey, large surface physical form more readily susceptible to enzymatic saccharification. There are several forms of pretreatment equipment available, including that developed by Stake Technology (Heitz et al. 1991). Others, such as the Wenger cooker-extruder, the St. Lawrence plug flow reactor and the conventional Masonite gun are described in Wayman and Parekh 1990. The vapour-cracker used in this work is a variation of the Masonite gun aimed at a more uniform control of the temperature and heat-up times during a treatment sequence. Its configuration is illustrated in Figure 1. It has the advantage of having a small size and yet high capacity. Its operation is described in the Experimental section.

In the work described here, waste paper was processed first in a mini-vapour-cracker located at the Université de Sherbrooke using several different sets of conditions. The resulting fibres were sent to Wayman's laboratory at the University of Toronto, where they were saccharified with enzymes and resulting sugars fermented to ethanol. Waste paper was chosen for this work because of current interest in municipal solid waste, of which waste paper is a

major component. The disposal of municipal solid waste in landfill creates undesirable environmental and political problems. It has been estimated (Wayman et al. 1991) that the diversion of the paper content of municipal solid waste to ethanol production would reduce the volume of municipal solid waste otherwise destined for landfill to about half, doubling the life of present landfill sites. At the same time, upon conversion to ethanol there is enough waste paper in, garbage to displace 10% of the gasoline now used by North America's motorists. Since ethanol is a relatively clean burning, non-petroleum, high octane motor fuel, its blending with gasoline has distinct air pollution advantages.

Since the manufacture of paper includes high temperature treatment of the wood feedstock, fairly good yields of ethanol are obtained without pretreatment ahead of saccharification-fermentation of waste paper. These yields, in the range of 350-400 liters of ethanol per tonne of waste paper, are, however, only 70-80% of the theoretical yields. It therefore appeared to be worthwhile to investigate the possibility of higher yields via pretreatment, and the mini-steam-cracker presented an attractive opportunity for such a study.

Experimental

Waste office paper (WOP) was shredded and a non-uniform feedstock having as characteristic dimensions strips ranging from 0.2-2 cm width and 2-6 cm length was produced. It was essentially at the equilibrium moisture content of the storage room (6.8% by weight). This feedstock was further pretreated in two different ways prior to the steam treatment:

(a) about 1 kg of the feedstock was soaked in pure water under vacuum until the cellulosic material was saturated with water and any occluded air removed; and

(b) another 1 kg of the feedstock was contacted with a solution of sulphuric acid 0.5% by weight, also under vacuum.

The consistencies used in the soaking/impregnation routines were 5% by weight of solids. The suspensions were drained and the wet material having typical moisture contents of about 70% was divided into fractions weighing 400-500 g. These fractions were kept cold (4°C) before their introduction into the reactor where the steam treatment is conducted.

Design and Operation of the mini-vapour-cracker

The reactor assembly used, named mini-vapour-cracker, is shown in Figure 1. It consists of a 4 liter autoclave being preheated by steam circulating internally and through its jacket which ensures a uniform temperature. Once the autoclave temperature is stable at the desired level, the material to be treated is introduced into the autoclave after opening the top closure. The latter is rapidly put back in, the autoclave is then sealed and saturated steam is introduced at the desired pressure. A thermocouple installed at the center of the bottom third of the autoclave measures the temperature of the wet material as it rises to the temperature of the steam. The signal from the thermocouple is recorded via a data acquisition system.

After a prescribed steaming time, the discharge blow valve is suddenly opened by a pneumatically-operated actuator and the treated material is ejected from the autoclave into a recovery vessel containing a prescribed amount of fresh water (1 liter in our experiments). A suspension is thus formed. The suspension is manually transferred into a glass container and the recovery vessel is rinsed with water to recover the material sticking to the walls. The rinse is added to the suspension. The latter is then vacuum filtered and the cake and the filtrate are stored at 4°C until further processing or analysis. The moisture content of the filtered material after vacuum filtration is between 62 and 74% by weight of the cake. The yield of fiber (g of dry recovered fiber per 100 g of dry feedstock) was consistently above 90%, and above 95% at treatment temperatures equal to or lower than 200°C. The treated material is well defibered and can be directly subjected to enzymatic hydrolysis. Alternatively, a mild refining

step can be introduced to totally defibrate the treated material and improve the contact between the fibers and the enzymes.

All the runs reported here held the fibres at the prescribed temperatures for 2 min. Temperatures ranged in 10°C steps from 170°C to 220°C. The treated fibres were packed in ice and dispatched to the University of Toronto for saccharification and fermentation.

Saccharification and Fermentation

The saccharification medium contained, per liter, 7 g cellulase (Multifect S-850, Finnish Sugar Company, 344 IU/g), 3mL cellobiase (Novozym 188, Novo, 91.5 IU/mL), trace elements, a small amount of Vitamin B12 and 2 mL Triton X100, and the pH was adjusted to 4.8. Saccharification was carried out in 250 mL shake flasks containing 100 mL saccharification medium, on a rotary shaker at 150 rpm, assisted by a few 8 mm stainless steel balls. To 100 mL of this saccharification medium was added 5 g of a steam-treated sample, and the suspension was incubated at 45°C for 6 h. The flask was then cooled to 37°C and to it was added 1 g Fleischmann's yeast (Saccharomyces cerevisiae) and 5 mL YMP (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone). Anaerobic closures were attached and fermentation proceeded on a rotary shaker at 150 rpm in an incubator at 37°C. Samples were taken at 24 and 48 h, and analysed for ethanol and residual sugars.

Analysis

Ethanol was determined by GC, Hewlett Packard 5700A, with ionization flame detector, using 0.7% n-propanol as internal standard. Residual sugars were determined by HPLC, Waters Sugar Analyser 1, with an Aminex HPX 87P column using water at 65°C as the eluant, a refractive index detector and 1.6% n-propanol as internal standard.

Results

The results are shown in Tables 1, 2, 3 and 4. The WOP contained 88.1% polysaccharides, out of which 69.7% was glucan, 14.4% xylan and 4.0% arabinan and mannan, the remainder being acid insolubles and moisture. We calculate the ethanol potential of this paper upon saccharification and fermentation by S. cerevisiae to be based on glucan plus 1/2 (arabinan + mannan) = 71.7 x 1.1 x 0.505 x 10/0.79 = 504 liters of ethanol per tonne.

The values for the control given in Tables 1 and 2 refer to WOP without any steam or other pretreatment ahead of saccharification and fermentation. They amount to 322 L ethanol per tonne of paper after 24 h fermentation, and 357 L/tonne after 48 h. The results in Table 1 show clearly that the water soaking pretreatment and then the steam treatment for 2 min at these temperatures made very little difference to ethanol yields. At temperatures of 200°C and higher there may be a 10% increase in yield, but considering the complexity of the total procedure this may not be significant.

The results in Table 2 show that pretreatment with 0.5% sulphuric acid improved ethanol yields substantially, from 357 L ethanol per tonne of WOP for the control to 460 L/tonne in 48 h fermentations, an increase of almost 30%. Indeed, while the control ethanol yield in 48 h fermentations was only 71% of the theoretical yield, the yields under the best conditions in Table 2 were 91% of theory. The results in Table 2 also demonstrate that even at temperatures well below 200°C the benefits were significant: ethanol yields in 48 h fermentations being 83% of theoretical. Furthermore, the improvement in ethanol yields above the control was quite noticeable in the 24 h results. The 24 h fermentation yields were all better than the 48 h control even at the lower temperatures. At the higher temperatures, the 24 h

fermentation yields were 95% of the 48 h yields, suggesting that the marginal value of the longer fermentations may not be justified under the conditions of Table 2.

Table 3 reports residual sugars in the medium following fermentation by S. cerevisiae for the series of fibers which were water-soaked before steam treatment. There were no significant differences in the amounts of residual sugars below 220°C. At 220°C there was higher cellobiose and glucose, while xylose and galactose were somewhat lower. In total, about 6% of the fiber remains in solution as sugars after fermentation, the range being 5.5 to 10.0. The highest value is at the highest temperature, although otherwise no trend is apparent.

The results shown in Table 4, and which refer to residual sugars after fermentation of the sulphuric acid impregnated and steam treated waste paper, demonstrate a trend, residual sugars being 5.7% of the fiber at 170°C and then decreasing to 1.6% at 220°C. Xylose and galactose in particular were lower. This suggests that at these processing conditions the xylose and galactose were probably dehydrated to furfural and hydroxymethyl furfural.

The filtrates taken following treatment in the mini-steam-cracker were analysed. There were no sugars in the samples which had been water soaked, while in those which had been soaked in 0.5% sulphuric acid the dissolved sugars, mostly xylose, amounted to 3 to 4% of the fiber.

While pretreatment with 0.5% sulphuric acid is shown here to be beneficial, in a previous study gaseous sulphur dioxide was added to a Stake reactor (Wayman et al. 1986), and the resulting fibers were readily saccharified by enzymes. The replacement of sulphuric acid by gaseous sulphur dioxide added directly to the reactor would result in superior contacting, in situ formation of sulphuric acid, and a simpler process. In the present work the appropriate equipment to introduce gaseous sulphur dioxide into the reactor was not available.

Conclusion

The vapour-cracker has been shown to be a simple and reliable equipment for pretreatment ahead of saccharification and fermentation of biomass, waste paper in this example. When impregnated with 0.5% sulphuric acid, waste paper steam treated at 170 - 220°C gave yields of ethanol 23% higher than controls with no pretreatment, upon saccharification by enzymes and 48 h fermentation by bakers' yeast. At temperatures above 200°C the ethanol yields approached the theoretical. At these higher temperatures, nearly all the benefits were observed in the first 24 h of fermentation, ethanol yields being 95% of 48 h yields. This suggests that the marginal value of the longer fermentations may not be justified. The suggestion is also made that direct addition of gaseous sulphur dioxide to the vapour-cracker in place of the sulphuric acid pretreatment would simplify the process.

Acknowledgements

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The original idea of developing an "improved steam explosion gun" is due to Ralph P. Overend who designed the first steam cracker constructed in our laboratory and whose steam circuit and discharge geometry and hardware have been progressively modified until the current configuration was established.

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Table 1: Steam Treatment of Waste Office Paper (WOP) in the vapour-cracker for 2 min, after Presoak in Water (Under Vacuum).

No.	Temperature	Ethanol, L/tonne 24 h	48 h
Control, no pretreatment		322	357
1.	170	303	367
2.	180	301	354
3.	190	303	359
	average 1-3	302	360
4.	200	327	375
5.	210	324	380
6.	220	324	372
	average 4-6	325	376
	average 1-6	314	368

Table 2: Steam Treatment of Waste Office Paper (WOP) in the vapour-cracker for 2 min, after Pre-soak in 0.5% Sulphuric Acid (Under Vacuum).

No.	Temperature	Ethanol, L/tonne 24 h	48 h
Control, no pretreatment		322	357
1.	170	391	402
2.	180	352	430
3.	190	358	428
	average 1-3	367	420
4.	200	416	460
5.	210	450	468
6.	220	448	453
	average 4-6	438	460
	average 1-6	403	440

Table 3: Residual Sugars after Saccharification and 48 h Fermentation of Waste Office Paper (WOP) Steam Treated in the vapour-cracker after Pre-soak in Water (Under Vacuum), in g/L.

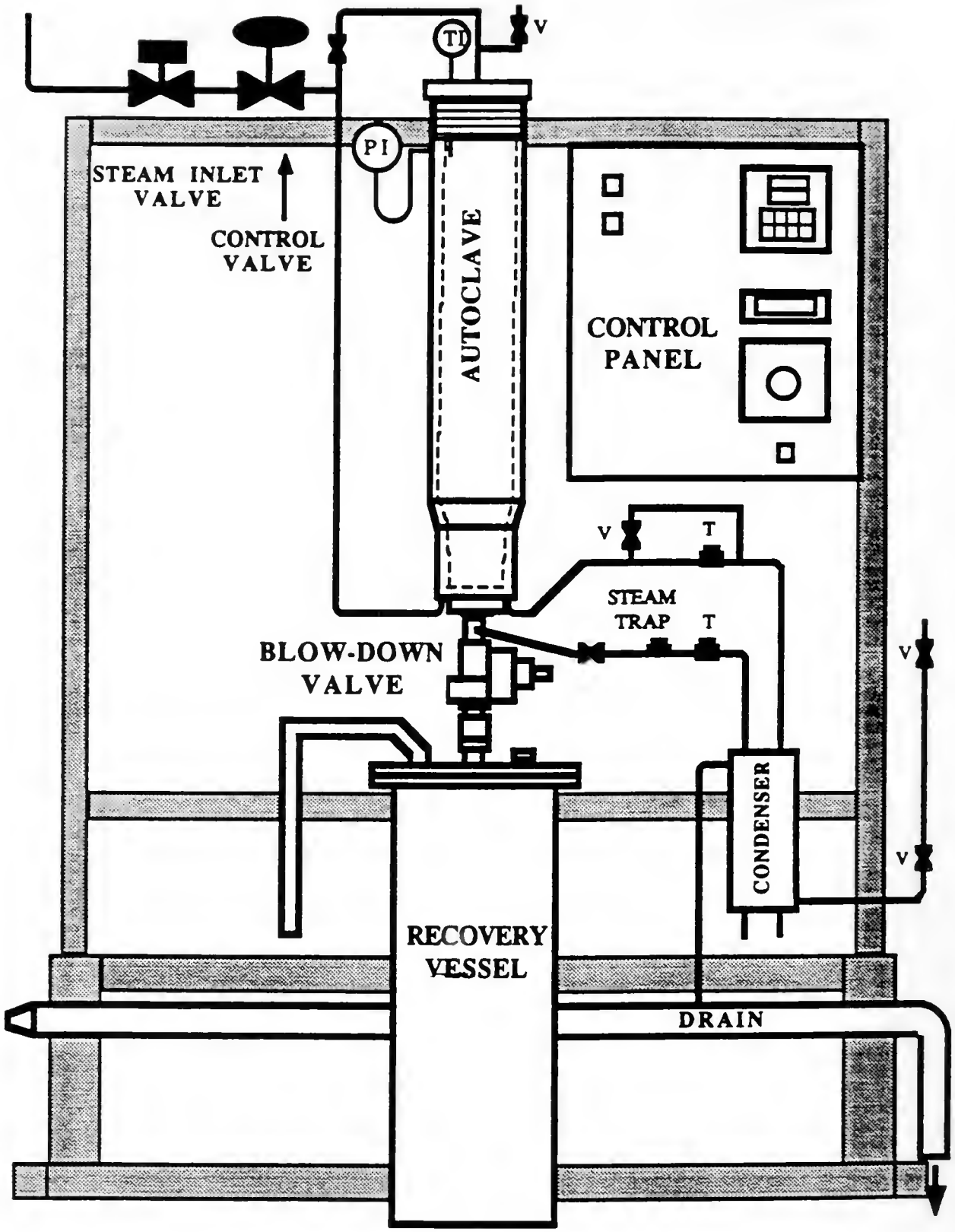
No.	Temperature	Cellobiose	Glucose	Xylose	Galactose
1.	170	0.33	0.0	1.27	1.46
2.	180	0.48	0.0	1.19	1.67
3.	190	0.0	0.0	1.34	1.39
4.	200	0.32	0.0	1.16	1.42
5.	210	0.38	0.0	1.30	1.15
6.	220	3.03	0.32	0.85	0.81

Table 4: Residual Sugars after Saccharification and 48 h Fermentation of Waste Office Paper (WOP) Steam-treated in the vapour-cracker after Pre-soak in 0.5% Sulphuric Acid (under Vacuum), in g/L.

No.	Temperature	Cellobiose	Glucose	Xylose	Galactose
1.	170	0.54	0.32	0.84	1.15
2.	180	0.32	0.0	0.67	0.81
3.	190	0.47	0.0	0.63	0.73
4.	200	0.28	0.12	0.14	0.37
5.	210	0.40	0.0	0.0	0.28
6.	220	0.38	0.0	0.41	0.0

Figure Caption

Figure 1. Mini-vapour-cracker used for the pretreatment of lignocellulosic materials.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CONDENSER
RECOVERY VESSEL
DRAIN

APPENDIX A-5 "Bioconversion of Refuse Derived Fuel to Ethanol"

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BIOCONVERSION OF REFUSE DERIVED FUEL TO ETHANOL

Morris Wayman* and Kim Doan

*Dept. of Chemical Engineering and Applied Chemistry
University of Toronto, Toronto, Canada M5S 1A4
phone: (416) 978 4905; FAX: (416) 978 8605

SUMMARY: Refuse Derived Fuels from 3 sources were converted to ethanol by enzymatic saccharification and fermentation. Two US RDFs yielded about 300 L ethanol/metric ton RDF, over 80% of theory, the others somewhat less. Potentially, RDF can make a substantial contribution to ethanol motor fuel supply.

INTRODUCTION: After removal of metals, ceramics and compostables in equipment for the fractionation of municipal solid waste, the residual light fraction is mostly paper and plastics, and has a high enough calorific value to be used as a fuel, Refuse Derived Fuel (RDF). The cellulose content is high enough to make RDF a candidate for the production of the environmentally friendly, high octane non-petroleum motor fuel ethanol. RDF includes contaminants which would be expected to inhibit enzymatic saccharification and fermentation. The purpose of this paper is to report the nature of the contaminants, the summative composition of several industrial samples of RDF, and the efficiency of conversion to ethanol.

EXPERIMENTAL: Following coarse primary shredding, the refuse fractionation systems employ screening, air classification and magnetic separation producing three or more fractions: metals, a heavy fraction containing compostables and ceramics such as sand and glass, and a light fraction mainly paper and plastics. Aluminum is also separated. The light fraction undergoes secondary screening and is pelleted or baled as RDF.

Samples of industrial RDF were supplied by Lundell Manufacturing Company, Inc., Cherokee, Iowa; NEC = National Ecology Co., a Babcock & Wilcox company, Timonium, Maryland; and WMI = Waste Management Inc., Oak Brook, Illinois (sample from BRINI process, Sundsvall, Sweden). These were mostly produced in 2 forms, a coarsely divided form (fluff) in pieces about 5 x 5 cm, or as cylindrical pellets 2 cm diameter by 3-10 cm long.

Summative analysis was carried out by treating the samples with 72% sulphuric acid at room temperature for 2 h (Wayman and Parekh, 1986). The resulting suspension of insolubles in the solution of oligomers was diluted with 2 L water (with cooling) and heated to 120°C for 1 h. After filtering, the sugar solution was brought to pH 5 with $CaCO_3$, filtered and analysed by HPLC. From the glucose concentration, the cellulose content of the sample was calculated.

Samples of RDF were shredded, saccharified and fermented. The saccharification medium contained, per L, 7 g cellulase (Multifect S-850, Finnish Sugar Company, 344 IU/g), 3 mL cellobiase (Novozym 188, Novo, 91.5 IU/mL), trace elements, Vitamin B and 2 mL Triton X100, and the pH was adjusted to 4.8. Saccharification was carried out in 250 mL shake flasks on a rotary shaker at 150 rpm, assisted by a few 6 mm stainless steel balls. To 100 mL of this saccharification medium was added 5 g shredded RDF, and this was incubated at 45 C for 6 h. The flask was then cooled to 37 C, and to it was added 1 g Fleischmann's yeast (Saccharomyces cerevisiae) and 5 mL YMP (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone). Anaerobic closures were attached and fermentation proceeded on a rotary shaker at 150 rpm in an incubator at 37 C. After 24 h, a further 5 g RDF plus an additional 0.7 g cellulase + 0.3 mL cellobiase were added and fermentation was continued. Samples were taken at 48 and 72 h and analysed for ethanol (GC) and residual sugars (HPLC).

Contaminants were determined qualitatively by careful picking apart and visual examination.

RESULTS:

Table 1. Contaminants in RDF

	Lundell	NEC
Paper*	++	++
Plastics	++	++
Aluminum foil	++	++
Wood	–	+
Cloth	–	++
Styrofoam	+	+
Sand, dirt	++	++
Metal	–	+
Hair	–	+

*Samples contained newspaper, white bond, computer paper, boxboard, corrugated, kraft and coated wrapping papers. The NEC sample contained tissue.

Table 2. Ethanol from RDF

RDF	cellulose %	ash %	ethanol L/metric ton found	theory	% of theory
Lundell pellets	53.5	7.3	316	376	84
Lundell fluff	52.6	3.8	-	370	-
NEC fluff	49.7	10.3	287	350	82
WMI pellets	48.6	13.9	244	340	72
WMI fluff	54.1	9.0	218	380	57

DISCUSSION AND CONCLUSIONS: The Lundell and NEC RDF samples gave the best yields, 84 and 82% of theory. The WMI RDF samples are quite diverse, with 72 and 57% of theoretical yield. Fermentations were all slow, about 72 h.

The amount of ethanol obtained from these samples of RDF varied widely, from a high of 316 L ethanol/metric ton of Lundell RDF to a low of 218 from a WMI RDF. The two Swedish (WMI) RDF samples gave yields fairly close together, 230 $\pm$ 13 L ethanol/metric ton.

Saccharification and fermentation are apparently inhibited by the contaminants, but the identity of the inhibitors has not been established. The one thing we can be sure of from these samples is that considerable variability can be expected in bioconversion of RDF, with yields not obviously related to cellulose or ash content.

Lundell plants are based on modules of about 100 metric tons/day of refuse, while the NEC and WMI (BRINI) systems

have much larger units, in the range of 1,000-2,700 metric tons refuse/day. The largest of these, the NEC RDF plant in West Palm Beach, Florida, processes 560,000 metric tons refuse/annum. Of this, about 75% is recovered in the light fraction, resulting in 420,000 metric tons/annum RDF. From the present results, the bioconversion of this RDF to ethanol would produce 120,000 cu m (32 million U.S. gallons) ethanol/annum. Such RDF plants can, thus, make a significant contribution to ethanol motor fuel supply.

The amount of enzyme used in these experiments, about 20% of the weight of RDF, was chosen so that enzyme availability was not the limiting factor in ethanol production. Optimization of the amount of enzyme required is the subject of a separate study. Ethanol concentrations were about 4% by volume, a marginally acceptable value but somewhat lower than a widely accepted minimum of 6% ethanol by volume for efficient distillation.

ACKNOWLEDGEMENT

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APPENDIX A-6 "Cellulase Production Induced by Carbon Sources Derived from Waste Newspaper"

Process Biochemistry 26 (1991) 93–100

Cellulase Production Induced by Carbon Sources Derived from Waste Newspaper

Shu Chen & Morris Wayman

Department of Chemical Engineering and Applied Chemistry, University of Toronto, Ontario, Canada M5S 1A4.

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Inducers derived from waste newspaper (or discarded office paper) were investigated for their use in cellulase production by T. reesei. *Partially enzymatically saccharified newspaper was found to be most effective. When 1·0 g newspaper and 0·07 g sorbose were used for cellulase production, the cellulases so produced after 6 days of fermentation saccharified 8·0 g waste white office paper in 9·34% suspension, resulting in 5·4 g fermentable sugars, or 6·3 g fermentable sugars with supplementation of β-glucosidase. Additional cellulases were produced from the cells and residual newspaper separated from the sixth day fermentation broth, and were effective in saccharifying another 8·0 g office paper. A total yield of 10–11 g fermentable sugars was obtained, from a total of 16 g office paper, being saccharified by the cellulase induced by 1 g partially saccharified newspaper.*

Effects of metabolizability of the inducers and addition of sorbose on cellulase production, kinetic behaviour of cellulase production, and comparison with commercial cellulases are presented.

INTRODUCTION

Cellulases produced by fungi of the *Trichoderma* genus, particularly *T. reesei*, have a high practical potential.[1,2] These enzymes remain too expensive to be used on a commercial scale for the conversion of waste lignocellulosics to liquid fuels, especially ethanol, although the practice has become increasingly important due to oil shortages and the environmental impact of petroleum based fuels. The major cost factor in cellulase production has been estimated to be the carbon source[3] since most of the cellulase preparations have been based on expensive substrates such as Solka Floc,[4–7] cotton,[8] Avicel,[9] carboxymethylcellulose (CMC)[9] and commercial cellulose pulp.[10] An effective approach to lowering the cost factor of the carbon source is to use less expensive substrates. Recent studies have shown that natural cellulosic biomass such as wheat straw,[10–12] bagasse[13] and aspen wood[14,15] are promising substrates for cellulase production. However, the utilization of these materials is always accompanied by harsh pretreatment conditions using either chemicals such as alkali or physical treatments such as ball milling. Such pretreatment methods increase the cost of these carbon sources. Also, most of the literature reports have been based on filter paper assay to evaluate the enzyme preparation, a method which may not reflect potential saccharification performance.[16]

The objectives of the present study were to evaluate inducers derived from waste newspaper for cellulase production by *Trichoderma reesei*, as

Corresponding author: Dr S. Chen. Telephone: (416) 978-4905; Fax: (416) 978-8605.

evaluated by saccharification performance of the resulting cellulase preparations, and to develop a practical approach to the production of less expensive but more effective cellulases.

MATERIALS AND METHODS

Substrate preparation

Partially saccharified newspaper (or office paper) was obtained by enzymatic hydrolysis of shredded newspaper (or office paper) using 2·3% cellulase (protein basis, on dry substance) at 6% solids, 50 °C, pH 4·8 and on a rotary shaker at 150 rpm for 48 h (or 24 h for office paper).

Cellodextrins were prepared from newspaper by acid hydrolysis, 10 g shredded paper being treated with 100 ml of 72% sulphuric acid at room temperature for 2 h. The resulting solution was then diluted to 2 l with cold distilled water, and neutralized with calcium hydroxide to a pH value of 5·0. The liquid was separated from the precipitate by filtration and concentrated to one-tenth of its original volume by vacuum evaporation.

NaOH-treated newspaper was prepared by soaking 10 g of shredded newspaper with 90 ml of 17·5% NaOH solution at room temperature for 18 h and by washing the soaked paper with sufficient water to remove the alkali.

Cellulase production

Trichoderma reesei Rut-C30, ATCC 56765, was obtained from the American Type Culture Collection, and grown on potato dextrose agar (Difco) slants at 30 °C for 3 days when sporulation occurred. The spore slants were stored at 4 °C until use. The organism was subcultured every month.

Inocula were prepared by transferring the conidia (10^6–10^7/litre) to the medium developed by Reese and Mandels,[1] modified with the addition of 10 g/litre glucose as carbon source and a concentration of peptone (Difco) of 1 g/litre. The fungus was grown in flasks for 48 h at 34 °C on a rotary shaker at 200 rpm.

A 10% (v/v) inoculum was used for initiation of cellulase production. The medium used was the same as that for growth except that the carbon source was 10–15 g/litre (on a carbohydrate basis) of specified substrates. The initial pH value was 5·0 and was not adjusted during fermentation. All the fermentations were carried out in 250-ml Erlenmeyer flasks containing 55 ml medium for 6 days (if not otherwise specified) at 28 °C, 150 rpm. At the termination of cellulase production, the pH value of the fermentation medium was adjusted to 4·8 with HCl or NaOH solution. The cellulase solution was separated from the mycelium by filtration, using Gooch crucibles with medium porosity, and used for saccharification or analysis.

Saccharification

Discarded used office paper was collected from the authors' Department, shredded by a Fellows Powershred 120, and used as a standard substrate for saccharification to examine the activity of the produced cellulase enzymes. A sample of refuse derived fuel (RDF) was obtained from commercial scale operations of BRINI in Sweden and also used as a substrate to check the ability of the produced cellulases to saccharify recycled waste paper. Saccharification was carried out by incubating 1·87 g (dry weight) of the shredded office paper (or 2·0 g of RDF) with 18·0 g of the cellulase filtrates in 50 ml flasks for 48 h at 50 °C, pH 4·8 and 150 rpm. At the end of saccharification, the samples were taken for sugar analysis.

The commercial cellulases used for comparative purposes were, Meicelase (donated by Meiji Seika Kaisha, Ltd., Tokyo, Japan), Multifect S-850, (the gift of Finnish Sugar Co. Ltd., Helsinki, Finland), Spezyme CE (kindly provided by Genencor International, Illinois, USA). β-Glucosidase, Novozyme 188 was purchased from Novo Industri (A/S, Copenhagen, Denmark).

Analytical techniques

Cellulase Filter Paper Units (IU/ml) were determined by the method of the International Union of Pure and Applied Chemistry (IUPAC).[16] Proteins in cellulase preparations were precipitated with 10% trichloroacetic acid (TCA) solution and quantified by the colorimetric method of Lowry *et al.*[17] Summative analysis of the paper materials (40 mesh) was conducted according to the method of Wayman and Parekh.[18] Monosaccharides and disaccharides were analysed by HPLC (Sugar Analyser 1, Waters) with an Aminex HPX-87P column using water at 65 °C as an eluent, refractive index detector and 16 g/litre n-propanol as the internal standard. Cellodextrins were characterized by the HPLC on an Aminex-42A column eluted with water at 85 °C. Mycelial (and cellulosic residue) dry weights were measured by filtering 30 ml samples through Gooch crucibles with medium porosity, washing with three volumes of

distilled water, and drying in a vacuum oven at 80 °C for 24 h to constant weight.

RESULTS AND DISCUSSION

Substrate analysis

Table 1 shows the composition of raw materials used in the present study. Acid insolubles in newspaper were mainly lignin, and in office paper and RDF, mainly ash. RDF also contained plastics which contributed to much of the other constituents.

The carbohydrate fractions of substrates used for cellulase production in the present study were characterized, as illustrated in Table 2. The carbohydrate fraction of the partially saccharified newspaper contained about 38% water soluble sugars, of which more than two-thirds was glucose, and about 62% water insoluble carbohydrates which were predominantly cellulose and hemicellulose. About 97% of the enzymatically saccharified office paper was water soluble; glucose amounted to about 70% and the other soluble sugars to about 27% of its carbohydrate fraction. Cellodextrins consisted of mainly glucose, oligomers (from DP2 to DP7) and hemicellulose sugars; each part being roughly one-third of the total. The calculation of substrate concentration used for cellulase production was based on the total carbohydrate content.

Effect of metabolizability of substrates on cellulase production

Generally, cellulose has been considered to be the best inducer for producing the whole spectrum of cellulase components.[2,3,19] The nature of the cellulose can greatly affect the inductive formation of the cellulase complex. Easily metabolizable substrates support rapid cell growth, give high productivity, but yield less powerful cellulases, while poorly metabolizable substrates are not broken down sufficiently readily to support adequate growth and give low productivity but may yield more powerful cellulases.[2] The 'best' substrates may be a compromise, having intermediate metabolizability.

Different substrates derived from newspaper with very different metabolizability were examined for cellulase production, with results as shown in Figure 1. Newspaper cellodextrin and partially saccharified newspaper produced a reasonable level of protein: 2·19 and 2·03 g/litre respectively, an indicator of cellulase formation. Cellulase activities were higher when partially saccharified newspaper was used as substrates than with cellodextrin as substrate, as illustrated by the saccharification results. NaOH-treated newspaper produced only 0·32 g/litre pro-

Table 1. Composition of Raw Materials

Sample	*Moisture* (%)	*Polysaccharides*	*Acid insolubles*	*Others*	*Glucose*	*Xylose*	*Galactose*	*Mannose + Arabinose*	*Total potential sugars*
Newspaper	8·89	70·86	26·89	2·25	54·19	8·60	2·49	12·67	77·95
Office paper	6·56	85·62	8·62	5·76	75·94	15·27	Not detected	2·97	94·18
Refuse Derived Fuel (BRINI)	5·16	66·62	16·04	17·34	51·66	6·48	0·94	5·20	73·28

Note: Unit of the composition (except moisture) is % of dry substance

Table 2. Composition of Carbohydrate Fraction of Substrates

Sample	*DP 1*	*DP 2*	*DP 3*	*DP 4*	*DP 5*	*DP 6*	*DP 7*	*Xylose*	*Galactose*	*Mannose + Arabinose*	*Water insoluble carbohydrates*
Cellodextrin	35·17	17·88	7·60	3·80	2·06	0·92	0·50	11·93	4·82	15·31	
Saccharified newspaper	27·19	2·69	0·39	0·48				3·54	0·29	3·58	61·84
Saccharified office paper	69·85	8·79	1·27					15·42		1·80	2·87

Note: Unit of the compositions is relative percentage. DP 1: glucose; DP 2: cellobiose; DP 3: cellotriose; DP 4: cellotetraose; DP 5: cellopentose; DP 6: cellohexose; DP 7: celloheptose.

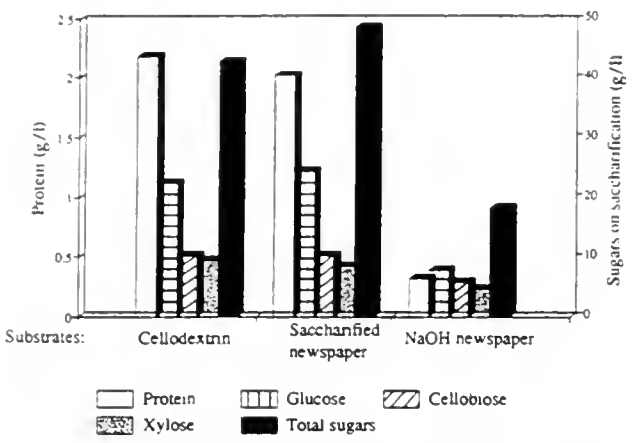

Fig. 1. Cellulase production induced by newspaper-derived substrates (15 g/litre) and saccharification performance of the cellulases.

tein which was far from sufficient for direct saccharification of 9·34 % office paper. Much of the NaOH-treated newspaper was found not to be utilized by the microorganism, indicating inadequate usefulness of the NaOH-treated newspaper for cellulase production.

The effect of substrate metabolizability on cellulase production was further investigated with the partially saccharified newspaper, its filtrate and its water washed residue, and saccharified office paper with addition of sorbose. Figure 2 illustrates that the partially saccharified newspaper was the most effective in terms of cellulase formation and saccharification ability of the resulting cellulases, followed by its filtrate and then its residue, while saccharified office paper was less effective for cellulase production. Successful cellulase production could be attributed to the complete spectrum of metabolizability of the substrate, being from soluble monosaccharides to crystalline cellulose. From the results, it can also be noted that the presence of lignin in the substrates did not interfere with cellulase production.

Effect of sorbose on cellulase production

Cellulases from *T. reesei* mutants are normally poor in β-glucosidase, one of the key components of the cellulase system, as indicated by the low ratio of glucose to cellobiose (10:7 to 10:4) in the saccharification media (Figure 1). This is because β-glucosidase is mainly cell wall bound and its release to fermentation supernatants results from lytic enzymes during conidiogenesis and from cell lysis.[20] L(-)sorbose has been reported to have a positive regulatory effect on the synthesis and secretion of β-glucosidase as well as cellulase since it inhibits glucan synthetase and hence the biosynthesis of glucan, the main component of the cell wall.[19,21]

As can be observed from Figure 1 (column group 2) and Figure 2 (column group 1), when *T. reesei* was cultivated on 10 g/litre of the partially saccharified newspaper with supplementation of 5 g/litre of L(-)sorbose at 96 h instead of 15 g/litre of the partially saccharified newspaper alone, extracellular protein increased by about 30 %, and both β-glucosidase activity and xylanase activity increased by as much as 45 % measured as the ratio of glucose to cellobiose and xylose content in the saccharification media.

Figure 3 illustrates that the time of sorbose addition was important. The earlier sorbose was added, the less cellulase protein was produced. Extracellular β-glucosidase activity always in-

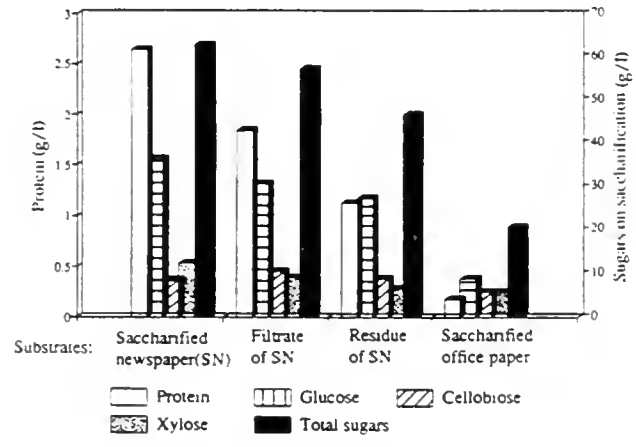

Fig. 2. Cellulase production from newspaper derived substrates (10 g/litre) with addition of sorbose (5 g/litre) at 96 h, and saccharification performance of the cellulases.

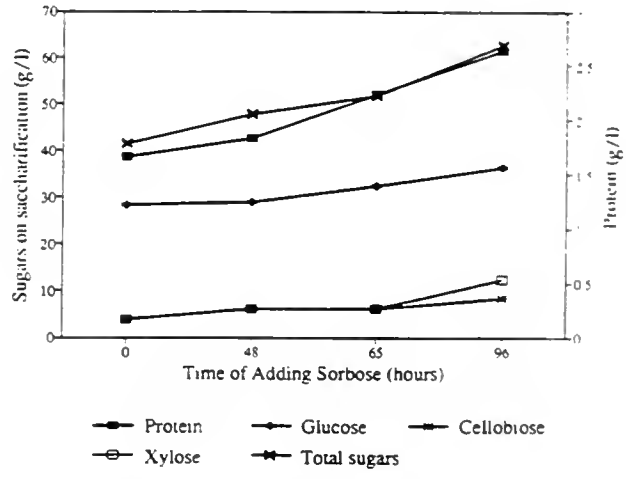

Fig. 3. Effect of time of adding sorbose on cellulase production and on saccharification performance of the cellulases. Partially saccharified newspaper: 10 g/litre: sorbose: 5 g/litre.

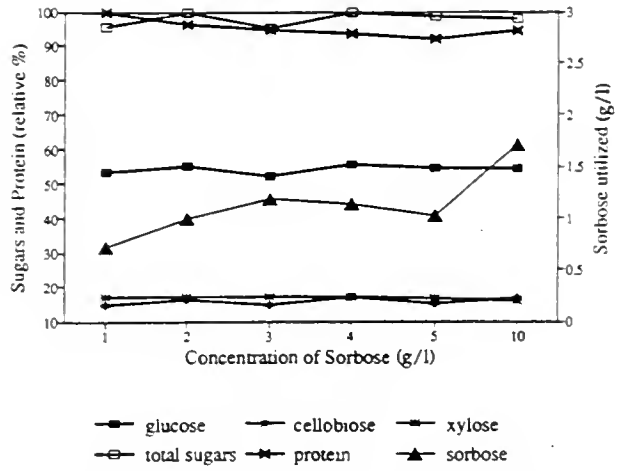

Fig. 4. Effect of concentration of sorbose on cellulase production and saccharification performance of the cellulases. Partially saccharified newspaper: 10 g/litre.

creased when sorbose was added regardless of the time of its addition.

In the present experiments, it was observed that about 75%–80% of the added sorbose was not consumed if it was added at 96 h. It was necessary to determine whether less sorbose could produce similar results to confer economic benefit. Figure 4 demonstrates the effect of sorbose concentration on cellulase production. It was rather surprising that sorbose concentration within the range of 1–10 g/litre made almost no difference to cellulase production. This may be explained as follows: since for a given fermentation system, the absolute amount of sorbose that *T. reesei* can metabolize does not significantly vary with sorbose concentration within a certain range, the regulatory effect of sorbose on cellulase production could be largely dependent on the metabolized portion of sorbose.

Kinetic behaviour of cellulase production

Figure 5 represents the kinetic behaviour of cellulase production on the partially saccharified newspaper (10 g/litre) with the addition of sorbose (5 g/litre) at 96 h. As shown in Figure 5(a), FPU of the culture filtrate increased at a roughly constant rate during the first 6 days and further increased at a lower rate during the next 4 days. Unlike the FPU profile, the protein profile showed a rapid increase in protein content during the first 4 days. More than 83% of the extracellular protein was accumulated during that period. This unparalleled nature between the two profiles shows that cellulases produced in the earlier stage had a lower specific activity (IU/mg protein), while cellulases produced in the late stage

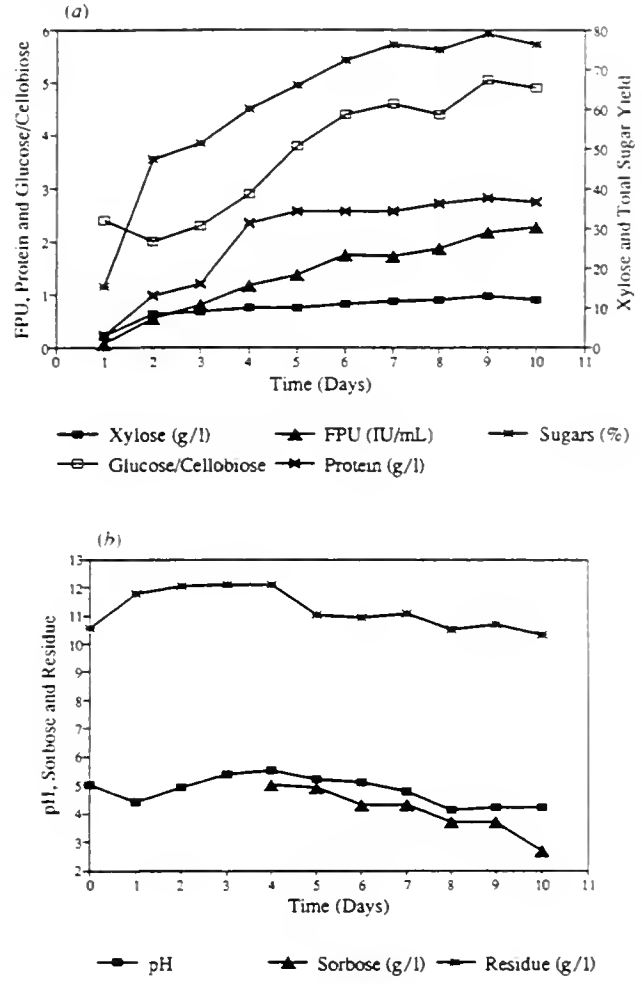

Fig. 5. Kinetic behaviour of cellulase production from partially saccharified newspaper (10 g/litre) and sorbose (5 g/litre added at 96 h).

had a higher specific activity. β-Glucosidase activity reflected by the ratio of glucose to cellobiose in the saccharification medium increased rapidly from the third day to the sixth day and levelled off at around the ninth day of cellulase production. More than 65% of xylanase activity reflected by the xylose content on saccharification was released within only the first 48 h, and the activity then rose at a much slower rate, levelling off at the ninth day. The overall saccharification ability of the culture supernatant, expressed as total sugar yield from the office paper, reached about 60% of the maximum value within the first 2 days, increased steadily in the next 5 days, and levelled off at the ninth day. The initial steep rise in the overall saccharification ability could be attributed to the stimulation of the xylanase activity in the filtrate. It should be noted that the overall saccharification ability of the culture filtrate was not reflected by its FPU, e.g. from the

third day to the sixth day, FPU more than doubled, while overall sugar yield increased by only 38%; FPU further increased after the ninth day, while sugar yield decreased after the ninth day. Therefore, FPU could not be used to predict potential saccharification performance of the enzyme preparations.

Figure 5(b) demonstrates the change of total solid residue, sorbose and pH of the fermentation medium with time. The solid residue included cell dry mass, lignin and residual carbohydrate polymer. The initial increase in the residue resulted from cell growth with the consumption of soluble sugars; the flat part of the profile could be attributed to cell growth with the consumption of insoluble carbohydrates; the declining residue content could reflect a decrease in the rate of cell growth and a slow consumption of residual carbohydrates. As already mentioned, much of the sorbose was not utilized by the cells even after 9 or 10 days of fermentation. The pH has been reported to have a significant impact on cellulase production with an optimum value of between 3·0 and 4·0 in the case of pure cellulose such as Avicel and Solka Floc as substrate[3] and higher in the case of natural cellulosic substrates.[10,22] The pH profile here is different from those reported (which were based on pure cellulose fermentations). When *T. reesei* is cultivated on pure cellulose the pH usually drops from more than 5 to as low as 2·5 after 2 days, then keeps stable at that level for a few days and rises slightly at the end,[23] so that strict pH control has been generally considered to be essential for cellulase production. The pH shown in Figure 5(b) was fairly stable, varying from 4·1 to 5·5, although no pH control was used. This would facilitate practical operations.

Comparison with commercial cellulases

A comparison of saccharification ability between commercial cellulases and cellulases produced in this study was made on the basis of the same dosage of protein used for saccharification. Sugar yields from saccharifying office paper by different cellulase enzymes are given in Figure 6. The total sugar yield was the highest, being 71·16% of the theoretical value, with the cellulases produced in this study. Glucose yield was higher than with these commercial enzymes, based on the same protein loading, except Meicelase. As for xylose yield, the cellulases obtained in this work were again the most effective. The 'home-made cellulases' showed lower β-glucosidase activity since more cellobiose was accumulated compared with the commercial cellulases.

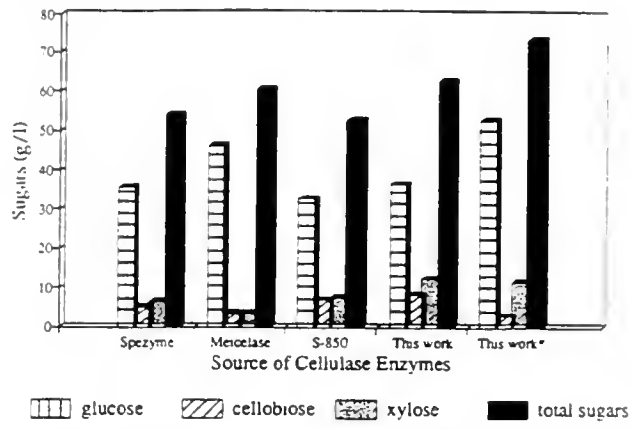

Fig. 6. Comparison in sugar yields from saccharification of 9·34% office paper by different cellulase enzymes. *With addition of 0·01 ml of β-glucosidase for each gramme of paper (dry).

However, this could be easily overcome by supplementation with 1% (1 ml/100 g substrate) of β-glucosidase. The home-made cellulases with addition of 1% β-glucosidase yielded 21%–39% more total sugars and 14%–62% more glucose respectively, compared with the commercial cellulases, on an equal protein basis.

From saccharification studies with different cellulase enzymes, it was again found that FPU was of little use in evaluating the practical saccharification performance of different enzyme preparations. As stated above, Figure 6 was obtained with the same enzyme loading in terms of protein. If the protein dosage was converted to FPU, the enzyme loadings became 37·8 IU of Spezyme, 25·9 IU of Meicelase, 21·5 IU of S-850 and only 17 IU of the home-made cellulases for each gram of paper. But the actual saccharification ability of the enzymes was not in the order of the FPU loading. For example, FPU loading of Spezyme was more than twice that of the home-made cellulase, while overall sugar yield and glucose yield from Spezyme were 87% and 98% of those from the home-made cellulases respectively. This indicated that the overall saccharification performance of cellulases produced in this study using partially saccharified newspaper with the addition of sorbose was better than or comparable to the commercial cellulases in spite of their lower specific activity (IU/mg protein).

Saccharification of refuse derived fuel (RDF)

The cellulase enzymes prepared as above were also tested for their saccharification ability on the RDF sample. From a 10% RDF suspension, 52·98 g/litre fermentable sugars, of which 28·55 g/litre was

glucose, was produced by the cellulase preparation. The overall saccharification efficiency was 72·3 % of the theoretical value. This can be further improved by the addition of β-glucosidase or perhaps by substrate pretreatment.

Cellulase production by recycled cells and residual substrates

In these experiments, fermentations were terminated at the sixth day of cellulase production. From the kinetic study, it was observed that the carbon source was not fully utilized by the sixth day of fermentation. Cells and lignocellulose residue were, therefore, separated from cellulase solution, mixed with fresh nutrient solution (no additional carbon source) and incubated for another 6 days under standard conditions. As anticipated, more cellulases (1·2 g/litre of protein) were produced and the cellulase supernatant yielded 52·82 g/litre fermentable sugars, of which 30·75 g/litre was glucose, from a 9·34 % suspension of office paper. These results illustrate that it was possible to prolong the period of cellulase secretion with a very limited amount of carbon source under the conditions used in this study.

CONCLUSIONS

Cellulases produced by *T. reesei* using 1 g of partially saccharified newspaper as inducer plus 0·07 g sorbose were effective in saccharifying 8 g waste white office paper, yielding 5·4 g fermentable sugars, or 6·3 g fermentable sugars when supplemented with a small amount of β-glucosidase. The economy of the process can be further improved by the recycling of *T. reesei* cells and residual carbon source. The cellulases produced from two successive cycles using a total of 1 g newspaper and 0·07 g sorbose saccharified 16 g of waste office paper, producing 10 g of sugars without supplementation of β-glucosidase or at least 11 g of sugars with supplementation of β-glucosidase.

Partially saccharified newspaper was an effective inducer of cellulase production by *T. reesei*. This probably resulted from the wide spectrum of its metabolizability. It is much less expensive than pure cellulose and is widely available. Its enzymatic treatment involved no harsh conditions which otherwise contribute to the additional cost of carbon sources for cellulase production.

Sorbose greatly enhanced cellulase production when it was added at the fourth day of fermentation. This effect was independent of sorbose concentration within the range of 1–10 g/litre.

Most of the cellulase was produced during the first 4 days. The overall production levelled off at around ninth day. Fairly stable pH profile of the cellulase production system required no strict control of pH during fermentation.

The use of saccharification performance on a substrate such as waste office paper, rather than that of FPU, represented a reliable way of evaluating different cellulase preparations. Cellulases produced from the partially saccharified newspaper with the addition of sorbose showed lower specific activity (IU/mg protein) and lower β-glucosidase activity but was of higher overall saccharification ability on waste office paper compared with commercial enzymes. Such cellulases were effective in saccharifying unpretreated RDF as received from a commercial municipal solid waste separation plant.

Research to improve β-glucosidase activity of the cellulase system and to increase the efficiency of cellulase production is continuing.

ACKNOWLEDGEMENT

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APPENDIX A-7 "Novel Inducers Derived from Starch for Cellulase Production by *Trichoderma reesei*"

Process Biochemistry 27 (1992) 327–334

Novel Inducers Derived from Starch for Cellulase Production by *Trichoderma reesei*

Shu Chen & Morris Wayman

Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A4

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Soluble inducers derived from starch were investigated for cellulase production by T. reesei. *Various methods of starch treatment were compared. Acid-hydrolysed starch was found to be most effective. When 1·0 g starch was employed for cellulase production, the cellulase so produced after 6 days of fermentation, with supplementation of 1% (0·01 ml/g paper) β-glucosidase, saccharified more than 15 g shredded waste office paper in 9·34% suspension, resulting in 10 g fermentable sugars, 72% of which was glucose. The effectiveness of the starch-derived inducers was compared with that of lactose, pure cellulose, CMC, xylan and prehydrolysate of pine wood. The effects of calcium chloride and sorbose addition on cellulase production, and the kinetics of cellulase production by the starch-derived inducers are presented.*

INTRODUCTION

Cellulases are inducible enzymes which are produced predominantly by fungi. The *Trichoderma* genus, particularly *T. reesei* contains many cellulolytic species.[1,2] Many carbon sources, either water soluble or water insoluble have been studied to determine the 'best' inducer. Cellulose has usually been considered to be the best inducer for producing a well-balanced cellulase system[2–4] but cellulose is insoluble in water. The rate of cellulase production is slow when insoluble cellulosic substrates such as pure cellulose[5,6] or pretreated waste cellulosic biomass[7,8] are used as the carbon sources. This low enzyme productivity increases the cost of cellulase enzymes. Insoluble cellulosic substrates also cause operational and rheological problems in bioreactors. The use of soluble carbon sources such as lactose,[9,10] cellobiose[11] and cellulose hydrolysate[12,13] for cellulase production allows high productivity, greater control of the fermentation and simplifies the operation of the process. Among soluble carbon sources, lactose has been considered the most attractive for its potential in cellulase production although it induces cellulase from *T. reesei* to a less extent than cellulose. It is however soluble in water up to 30% (w/w); it is a by-product of the cheese industry but is not as readily available as cellulose or glucose. Hydrolysis syrup produced from ball-milled cellulose by cellulase enzymes has been demonstrated to be comparable to lactose in terms

Corresponding author: Dr S. Chen. Tel: (416) 978 4905. Fax: (416) 978 8605.

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of its inductive ability for cellulase production, but pure cellulose is expensive, and ball milling and enzymatic saccharification increase its cost further.

In order to avoid the drawbacks of insoluble carbon sources and to address a practically feasible soluble carbon source, we have studied different soluble inducers derived from corn starch for cellulase production by *T. reesei*.

MATERIALS AND METHODS

Preparation of starch-based inducers

Corn starch, α-amylase (EC 3.2.1.1, Termamyl-120L®) and glucoamylase (EC 3.2.1.3, AMG-200L®), the products of Novo Industri, A/S, Copenhagen, Denmark, were kindly provided by St Lawrence Reactors Ltd (Ontario, Canada). Liquid β-glucosidase (8·95% protein), Novozym 188, was purchased from Novo Industri.

Starch slurry at a concentration of 10–30% dry solids was liquefied with 0·1% α-amylase (on dry solids) in a water bath at pH 6·8, 80–85 °C for 30 min under constant stirring, and autoclaved at 121 °C for 10 min for some of the preparations. The dextrins were then saccharified with 0·1–1·0% glucoamylase (on dry solids) at pH 5·0, 50 °C and on a rotary shaker at 150 rev/min for 48–89 h. The glucose syrup was (or was not) further incubated with 1·0–2·0% β-glucosidase (on dry solids) at pH 5·0, 50 °C on a rotary shaker at 150 rev/min for 48 h.

Alternatively, starch slurry at a concentration of 17·5–30% dry solids was hydrolysed with 0·3 N HCl in an autoclave at 121 °C for 1–2 h. The resulting syrup was chilled in a water bath and neutralized with calcium hydroxide to a pH value of 5·5.

Glucose syrups produced with enzymes were designated as ES1–ES6 according to different conditions, and those produced with acid designated as AS1–AS3. They were used as carbon sources for cellulase production.

Sources of other inducers

Cellulose Alpha-Floc was obtained from Brown Co., USA. Birchwood xylan was supplied by Sigma Chemical Co. Lactose and carboxymethyl cellulose (CMC) were supplied by BDH. Prehydrolysate of pine wood was extracted from prehydrolysed pine wood obtained by passing pine chips containing 2·6% SO_2 on wood through a Wenger extruder at 200 °C and a residence time of 45 seconds.[14]

Cellulase production

Trichoderma reesei Rut C-30, ATCC 56765, was obtained from the American Type Culture Collection. The procedures used for culture maintenance, inoculum preparation and cellulase production were as described before[7] except that the following modifications were made. The carbon source used for cellulase production was 13·5 g/litre (based on total reducing sugars) of specified substrates. The initial pH value for cellulase production was 5·5 and was controlled with 0·1 M KH_2PO_4 (if not otherwise specified).

Saccharification

The cellulase preparations from 6 day cultures were evaluated by their 48 h saccharification performance on shredded waste office paper in a 9·34% suspension as described before.[7]

Analytical techniques

Cellulase Filter Paper Units (IU/ml) and β-glucosidase activity (cellobiase unit as IU/ml) were determined by the methods of the International Union of Pure and Applied Chemistry (IUPAC).[15] Protein in cellulase preparations was precipitated with 10% trichloroacetic acid (TCA) solution and quantified by the colourimetric method of Lowry *et al.*[16] Analysis of the office paper was conducted according to the method of Wayman and Parekh.[17] Sugars were analysed by HPLC (Sugar Analyser 1, Waters) with an Aminex HPX-87P column using water at 65 °C as an eluent, refractive index detector and 16 g/litre *n*-propanol as the internal standard. Mycelial dry weights were measured by filtering 30 ml samples through Gooch crucibles of medium porosity, washing with three volumes of distilled water, and drying in a vacuum oven at 80 °C for 24 h to constant weight.

RESULTS AND DISCUSSION

Analysis of starch hydrolysates

The true induction mechanism of the *T. reesei* cellulase complex is not known. However, β-linked disaccharides of glucose have been considered to be responsible for the inducing properties of a cellulase production medium.[18,19] In particular sophorose (2-O-β-glucopyranosyl-D-glucose) was reported to be able effectively to induce cellulase enzymes when it was incubated with young washed mycelium of *T. reesei* QM6a even at a very low concentration (e.g. 0·5 g/litre).[19] Most of the disaccharides, especially

Table 1. Inducers prepared from corn starch by acid

Inducer	*Concentration (% dry solids)*	*Hydrolysis (°C, h)*	*Glucose (g/litre)*	*TRS[a] (g/litre)*
AS1	17·5	121, 1	172·67	190·01
AS2	17·5	121, 2	163·69	180·57
AS3	30·0	121, 1	239·45	303·88

TRS, total reducing sugars.

sophorose, are rare sugars and not widely available in nature. They can be prepared by reversion reactions such as condensation and/or transglycosylation of glucose by acid[20] or β-glucosidase.[21]

Under normal conditions for glucose production from starch the equilibrium of glucose and its reversion products in solution strongly favours glucose. Since the reversion products were wanted for cellulase production, various conditions which could shift the equilibrium towards these products were tested for the preparations of the starch hydrolysates used in the present study. Both acid and enzyme approaches were investigated. To increase the yield of reversion products, the concentration of starch, one of the most important factors, should be high; the acid should have high catalytic activity for condensation; the strength of the catalyst should also be high.[21,22] In addition, the considerations should include the practical feasibility of a process.

Tables 1 and 2 show the conditions employed for the preparation of the starch hydrolysates, and content of glucose and total reducing sugars (TRS) in the resulting starch-derived carbon sources used for cellulase production in the present study. Glucose was the principal sugar in all the preparations. The difference between TRS and glucose was contributed mainly by disaccharides which were formed from glucose by condensation and/or transglycosylation. The disaccharides were not identified in the present study. As shown in Table 1, the acid hydrolysates contained high levels of the reversion products, especially at high concentration of starch, being about 21% of the TRS when starch was hydrolysed in a suspension of 30% dry solids. A prolonged time for hydrolysis did not increase the level of reversion products but resulted in the decomposition of glucose. Compared with the acid hydrolysates, most of the enzyme hydrolysates contained less reversion products (Table 2). The use of β-glucosidase, high levels of glucoamylase and high concentration of starch resulted in an increase in the amount of reversion products in the hydrolysates. A liquefied 20% starch suspension saccharified for 72 h by 1% glucoamylase with the simultaneous addition of 1% β-glucosidase (ES3) resulted in the highest level of reversion products, being 11·5% of TRS.

Cellulase production on acid-hydrolysed starch

Different substrates produced from starch by acid hydrolysis under different conditions (Table 1) were examined as inducers for cellulase production in batch fermentations by *T. reesei* Rut C-30. Two controls with analytical reagent glucose and untreated starch were also set up respectively. The results are shown in Fig. 1. The acid hydrolysates were all effective for cellulose production, yielding a high level of soluble protein from 3·03 to 3·93 g/litre. When the cell-free culture filtrates were used directly to saccharify shredded waste office paper in a 9·34% suspension, 62·66–

Table 2. Inducers prepared from corn starch by enzymes

Inducer	*Concentration (% dry solids)*	*Liquefaction*	*Saccharification (with/without β-glucosidase treatment)*	*Glucose (g/litre)*	*TRS[a] (g/litre)*
ES1	10	80 °C, 30 min	1% glucoamylase, 48 h	103·38	105·81
ES2	10	80 °C, 30 min	1% glucoamylase, 48 h, 2% β-glucosidase, 48 h	98·29	107·31
ES3	20	85 °C, 30 min, 121 °C, 10 min	1% glucoamylase + 1% β-glucosidase, 72 h	192·64	217·64
ES4	20	85 °C, 30 min, 121 °C, 10 min	0·1% glucoamylase, 72 h	204·38	207·75
ES5	20	85 °C, 30 min, 121 °C, 10 min	1% glucoamylase, 72 h	214·66	223·86
ES6	30	85 °C, 30 min, 121 °C, 10 min	0·1% glucoamylase, 89 h	302·41	306·83

[a] TRS, total reducing sugars.

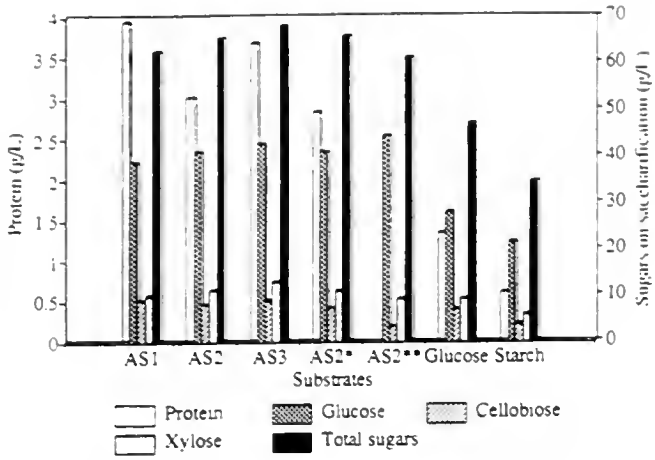

Fig. 1. Cellulase production on acid-hydrolysed starch and saccharification performance of the cellulases. AS2*: without the use of KH_2PO_4 for pH control during cellulase production. AS2**: 50% of AS2 with the addition of 1% β-glucosidase for saccharification.

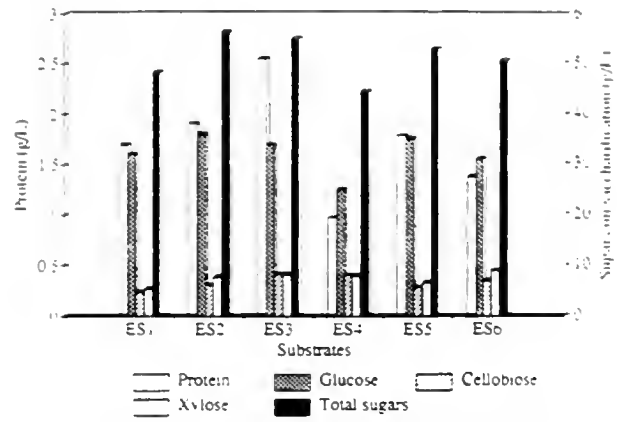

Fig. 2. Cellulase production on enzyme-saccharified starch and saccharification performance of the cellulases.

68·13 g/litre of total reducing sugars, as measured by HPLC, was produced. When the culture filtrate from AS2 was diluted to 50% of its original strength and then used for saccharification with the addition of 1% β-glucosidase (0·01 ml/litre for each gram of dry paper), the cellulases were similarly effective. This indicates that the cellulase activities, as judged by their saccharification performance, were high. As illustrated by the saccharification results, the cellulases were more active when the substrates used for the cellulase production were produced under conditions which favour the formation of condensation products although the differences in the activities were not large. Untreated starch and pure glucose produced only 0·60 g/litre and 1·34 g/litre protein respectively, and their resulting filtrates were not strong enough to carry out direct saccharification of 9·34% paper with an acceptable yield. Successful cellulase production on the acid-hydrolysed starch could be attributed to the presence of the condensation products in the substrates which were probably the real inducers of cellulase production. Increased levels (within a certain range) of the condensation products, therefore, resulted in higher activities of the culture filtrates.

Since the pH value for cellulase production in the present study was controlled by addition of 0·1 M KH_2PO_4 which is also a component of the nutrients, it was necessary to determine whether the additional salt enhanced cellulase production. As can be seen in Fig. 1, the results obtained with and without the additional salt were very similar. The effect of the additional KH_2PO_4 other than maintaining pH during cellulase production can therefore be excluded. When the additional KH_2PO_4 was not used, and the pH was not adjusted during cellulase production, the pH value of the fermentation broth remained around 3·5 suggesting that the acid hydrolysate of starch had high buffering capacity. This could be due to the formation of organic acids such as formic acid and acetyl propionic acid from glucose decomposition during heating at low pH.[22]

Cellulase production on enzyme-saccharified starch

Starch hydrolysates prepared by enzymatic saccharification under various conditions (Table 1) were also evaluated for cellulase production in batch cultures of *T. reesei* Rut C-30, with results as shown in Fig. 2. In all cases, enzyme-saccharified starch was not as effective as acid-hydrolysed starch for cellulase production. This was not surprising since the enzymic hydrolysates in general contained smaller amounts of transglycosylation products.

The enzymic hydrolysates produced under different conditions resulted in different results. The difference between ES1 and ES2 was that ES2 was prepared by incubation of ES1 with 2% β-glucosidase for 48 h. The treatment of the saccharified starch with β-glucosidase resulted in an increase in effectiveness of the substrate for cellulase production by more than 12% as judged by protein formation and the saccharification performance of the resulting cellulases. It appeared that β-glucosidase was responsible for the increased inducing ability of the starch hydrolysate, probably due to the formation of the reversion compounds, such as sophorose. However, the effect of glucoamylase on the increased inducing ability of the hydrolysate should not be excluded, since the enzyme remained active during incubation of the hydrolysate (ES1 -

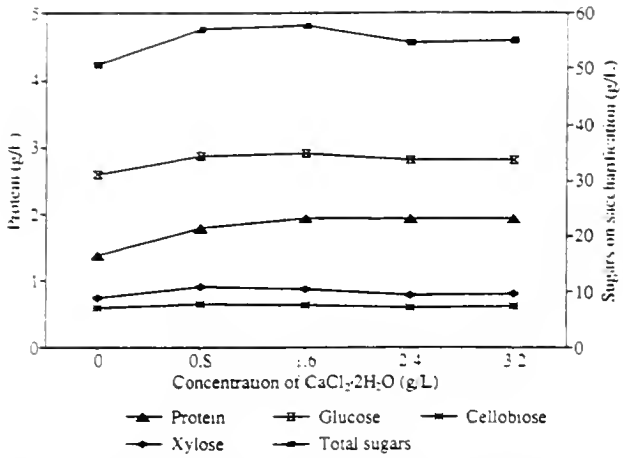

Fig. 3. Effect of calcium on cellulase production and on saccharification performance of the cellulases. Substrate: ES6.

with β-glucosidase. This can be judged by comparing the preparations as well as the results between ES3 and ES5. ES3 was prepared using 1% glucoamylase with the simultaneous addition of 1% β-glucosidase, while ES5 was prepared with 1% glucoamylase only. The use of β-glucosidase in ES3 more than doubled the content of reversion products, but only slightly enhanced the saccharification activity of the culture filtrate although it increased the formation of protein significantly. From the results with ES4, which was prepared with a reduced level of glucoamylase, it was apparent that a lower level of glucoamylase resulted in about a 16% decrease in the saccharification ability of the culture filtrate. When the hydrolysate (ES6) was prepared at a higher concentration (30% dry solids) and longer incubation time using the same low level of glucoamylase as ES4, the level of cellulase production was regained about 13% in terms of the saccharifying activity of the culture filtrate. These results demonstrate that glucoamylase was also responsible for the enhanced inducing ability of the starch hydrolysates. Since reversion compounds of glucose resulting from glucoamylase are essentially α-glucobioses,[21] there are probably inducers for cellulase production which are α-linked dimers of glucose.

Effect of calcium on cellulase production

In addition to the difference both in quantity and quality of reversion products between the acid-hydrolysed starch and the enzyme-saccharified starch, a significant difference between the two types of substrates was their calcium content. The acid hydrolysates contained high concentrations of calcium resulting from the use of lime for neutralization of pH which might have also served as a stimulatory factor for cellulase production. Studies on the effect of calcium on cellulase production were therefore conducted using enzyme-saccharified starch (ES6) as the substrate with the addition of various levels of calcium chloride. Calcium increased the protein level by up to 41·3% and the saccharification activity of the culture filtrates by up to 13·4% (Fig. 3). For effectiveness an optimal level of calcium of 1·6 g/litre calcium chloride dihydrate was added to the fermentation medium. The reason for the positive effect of calcium on cellulase production is not known. It is possible that calcium is responsible for some kind of change in the permeability of the cell wall, resulting in a more rapid excretion of the enzymes and this in turn improves cellulase synthesis.

Kinetic behaviour of cellulase production on acid-hydrolysed starch

It was essential to know the kinetic behaviour of cellulase production on a new substrate in order to understand the life cycle of the microorganism and of the features of the whole process in order to exercise better control over cellulase production. Figure 4a represents the time course of certain fundamental parameters of cellulase production by *T. reesei* on acid-hydrolysed starch AS3 under our standard conditions. Figure 4b illustrates the saccharification ability of the culture filtrates taken at different times of cultivation.

Metabolic activity, as measured by a decrease in pH, commenced immediately after inoculation of the fermentation medium. The exponential growth phase continued for about 3 days until more than 97% of the glucose was assimilated. The growth then shifted to a death phase although the data did not exclude the possibility that a very short stationary phase existed before the declining phase. The death phase may represent elimination of the carbon source for growth, secretion of cellulases and autolysis of mycelium.

The cellulase activity (assayed as FPU) of the culture filtrate was detected soon after the beginning of the cultivation. It increased slowly during the exponential growth phase, attaining about 30% of the final, then increased rapidly during the early stage (about 2 days) of the declining phase, and finally levelled off when the substrate was completely consumed. The increase in FPU during the early stage of the death phase may have resulted from both active secretion and cell lysis.

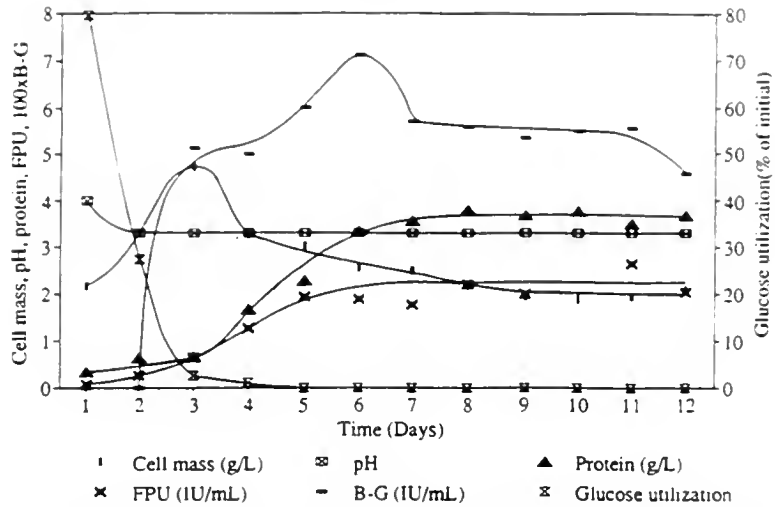

Fig. 4a. Kinetic behaviour of cellulase production on starch hydrolysate (AS3). FPU: filter paper units; B-G: β-glucosidase activity.

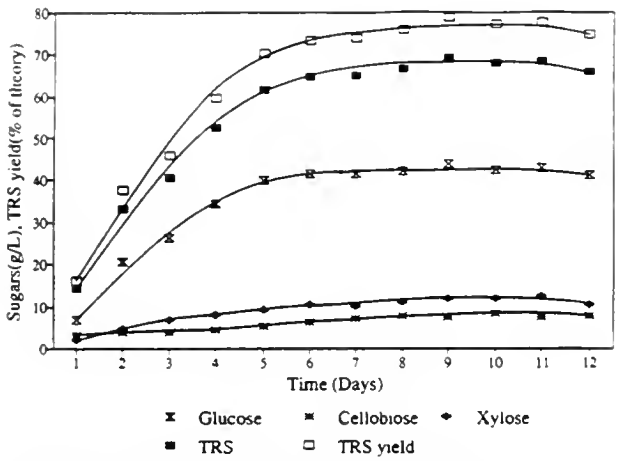

Fig. 4b. Kinetic behaviour of cellulase production on starch hydrolysate (AS3). TRS: total reducing sugars.

β-glucosidase is known to be mainly cell wall bound and its release into the culture filtrates results from autolysis.[23] Only minor amounts of β-glucosidase can be found in the culture filtrate during the early stages of cellulase production. The activity in the medium rises coincidentally with autolysis of the cells when the culture enters the stationary phase. As expected, β-glucosidase activity was hardly detectable at the beginning of cultivation but rose sharply after 2 days when the cells were growing, attaining about 72% of its highest value. The remaining β-glucosidase was produced after growth reached the maximum cell concentration level. The loss of β-glucosidase activity after the sixth day could be due to denaturation of the enzyme upon incubation at a pH value (3·3) lower than the optimum for β-glucosidase. The β-glucosidase activity (0·05–0·07 IU/ml) was very low in the medium, which could also have resulted from the lower pH since a much higher β-glucosidase activity (e.g. 0·2 IU/ml, results not shown) was possible when *T. reesei* was cultivated on an acid-hydrolysed starch at a higher pH.

The profile of extracellular protein was similar to that of FPU during the first 5 days of cellulase production, and then continued to rise until its maximum value on around the eighth day. The extra protein released after the fifth day did not make a significant contribution to FPU. The reason could be that some of the protein released from the autolysing cells was non-catalytic.

The overall saccharification ability of the filtrate, expressed as total sugar yield from the paper, increased linearly for the first 3–4 days and reached nearly 60% of the maximum value when the FPU was only about 30% of its maximum. This ability then increased at a reduced rate and levelled off on around the sixth to seventh day. Glucose and xylose formation by the cellulases in the saccharification media followed a pattern similar to that of the overall saccharification ability. It should be emphasized that the saccharification ability of the filtrates was not well reflected by the FPU. As reported earlier,[7] the saccharification performance of a culture filtrate may be a more reliable criterion than the FPU for the evaluation of a cellulase preparation for a specific application.

Comparison with other carbon sources

A comparison of inducing ability between a starch-derived carbon source, an acid-hydrolysed starch (AS3), and other carbon sources was made in batch cultures of *T. reesei* Rut C-30. Figure 5 illustrates

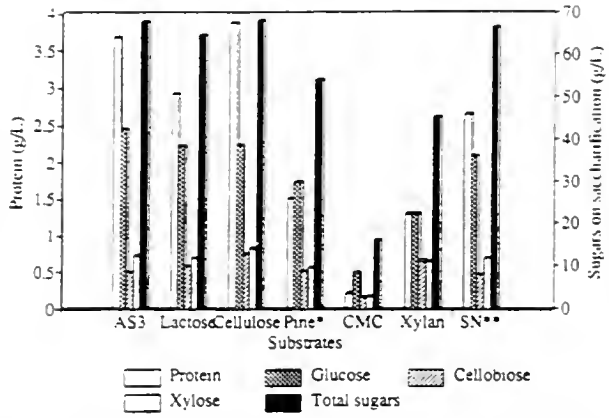

Fig. 5. Cellulase production on different carbon sources and saccharification performance of the cellulases. Pine*: pine pre-hydrolysate SN**: saccharified newspaper.[7]

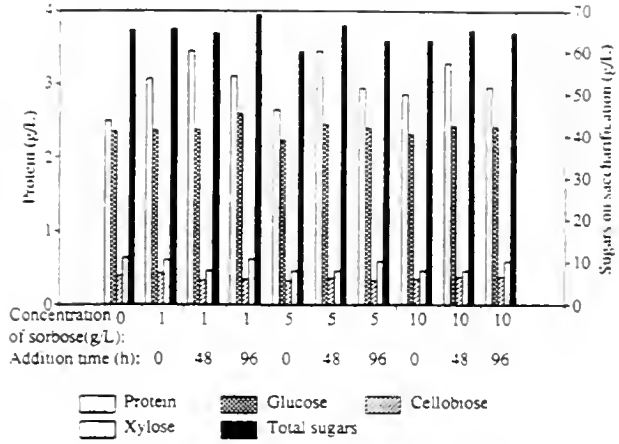

Fig. 6. Effect of sorbose on cellulase production and on saccharification performance of the cellulases. Substrate: AS2. Cellulase production was carried out at 26 °C.

the levels of soluble protein in the culture filtrates and the saccharification performance of the filtrates resulting from the different carbon sources. The most effective substrates, in terms of protein productivity and cellulase activity, were cellulose, AS3, partially saccharified newspaper and then lactose, while pine pre-hydrolysate, xylan and especially CMC, were much less effective. Although the protein produced from AS3 was slightly less than that from cellulose, the overall saccharification ability of both cellulases were similar. Compared with the cellulases resulting from induction on cellulose, the cellulases from AS3 showed higher β-glucosidase activity and lower xylanase activity as indicated by the glucose, cellobiose and xylose content in the saccharification media. The results showed that acid hydrolysates of starch were good carbon sources for cellulase production.

Effect of sorbose on cellulase production

In a previous study where pretreated newspaper was used as carbon source,[7] L(-) sorbose greatly enhanced cellulase production by *T. reesei*. The time of sorbose addition was important and its positive regulatory effect on cellulase production was independent of sorbose concentration within the range of 1–10 g/litre. It was expected that sorbose might have a similar enhancement effect on cellulase production by *T. reesei* in the present study using the starch hydrolysates as the carbon sources. A series of experiments was, therefore, carried out to evaluate the effect of sorbose on cellulase production using acid-hydrolysed starch AS2 as substrate and to determine the concentration of sorbose required for effectiveness and the most beneficial time of addition.

Figure 6 demonstrates the effect of sorbose on cellulase production. The addition of sorbose to the fermentation system during cellulase production increased the protein level in the culture filtrates in all the cases tested. The increase was most significant (up to 38%) when sorbose was added 48 h after initiation of cellulase production, regardless of the concentration of sorbose applied. However, in terms of the saccharification efficiency of the culture filtrates, the use of sorbose was not always beneficial. Only cellulases produced with the addition of 1·0 g/litre of sorbose at 96 h resulted in an appreciable (6·1%) increase in the saccharification ability of the culture filtrate, while those produced in the other cases had similar saccharification ability to the cellulases produced without the addition of sorbose. These results suggested that sorbose may not be a universal enhancer of cellulase production by *T. reesei* and that its effect on cellulase production may depend on the carbon source used.

CONCLUSIONS

Soluble starch hydrolysates prepared by 0·3 N HCl were powerful inducers of cellulase production by *T. reesei*. Cellulases produced by *T. reesei* using 1 g starch as carbon source were effective in saccharifying 15 g shredded waste office paper, yielding 10 g fermentable sugars when supplemented with a small amount of β-glucosidase.

The inducing ability of the acid-hydrolysed starch was comparable to that of pure cellulose. Since the hydrolysate is water soluble, the adoption of this carbon source would simplify the operation of

cellulase production and allow development of highly productive continuous processes for cellulase production. The carbon source is widely available and if whole grain instead of purified starch is used as a starting material, the raw material would be much less expensive than commercial cellulose.

The high inducing ability of the acid hydrolysates of starch could be attributed to the reversion products of glucose as well as the high calcium content of the hydrolysates.

Cellulase production was fast on the soluble substrates. Most of the cellulase was produced during the first 4 days and the overall production levelled off at around the sixth day.

Sorbose did not enhance cellulase production significantly when it was applied to the starch-based cellulase production system.

ACKNOWLEDGEMENT

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APPENDIX A-8 "Cellulase Production by *Trichoderma reesei* using Whole Wheat Flour as a Carbon Source"

Cellulase production by *Trichoderma reesei* using whole wheat flour as a carbon source

Morris Wayman and Shu Chen

Department of Chemical Engineering and Applied Chemistry
University of Toronto, Toronto, Ontario, Canada

Soluble inducers derived from whole wheat flour were investigated for cellulase production by T. reesei. *Acid-hydrolyzed wheat flour prepared using 0.3–0.6 M HCl proved to be very effective. The wheat flour hydrolyzates (13.5 g* l^{-1}*) yielded 2.52 IU* ml^{-1} *against filter paper and 0.28 IU* ml^{-1} *cellobiase activity. When 1.0 g wheat flour was employed for cellulase production, the cellulase so produced after 6 days of fermentation, with supplementation of 1% (0.01 ml* g^{-1} *paper) β-glucosidase, saccharified 13 g shredded waste office paper in 9.34% suspension, resulting in 9.6 g fermentable sugars, 71% of which was glucose. The feasibility of elimination of additional nutrients and elimination of pH control for cellulase production by* T. reesei *using wheat hydrolyzates as substrate was examined. Comparative studies using enzymatically liquefied wheat, whole wheat flour, wheat bran, and pure starch are also reported.*

Keywords: Cellulase; cellulase production; *Trichoderma reesei*; whole wheat flour; wheat hydrolyzate

Introduction

Cellulases produced by fungi of the *Trichoderma* genus, particularly *T. reesei*, have a high practical potential.[1,2] These enzymes remain too expensive to be used on a commercial scale for the conversion of waste lignocellulosics to liquid fuels, especially ethanol, although the practice has become increasingly important due to oil shortages and the environmental impact of petroleum-based fuels.[3] Studies of cellulase production have shown that the nature of the carbon source is a most important factor in determining the effectiveness and economy of the inductive production of cellulases.[4] Generally, pure cellulose such as Solka Floc, cotton, Avicel, or commercial cellulose pulp has been considered to be the best inducer for producing a well-balanced cellulase system with high yields of the enzymes.[2,4–6] However, pure cellulose is too expensive to be used for large-scale production, and its water-insoluble nature results in a low rate of cellulase production, and causes operational and rheological problems in bioreactors. Lignocellulosic wastes such as waste newspaper,[7] wheat straw,[8] bagasse,[9] and aspen wood[10] are inexpensive carbon sources, which must be properly pretreated to be suitable for cellulase production. These insoluble substrates increase the operational and rheological problems in bioreactors.[10] The use of soluble carbon sources such as lactose,[11,12] cellobiose,[13] and cellulose hydrolyzate[14] for cellulase production allows greater control of the fermentation and simplifies the operation of the process. Among soluble carbon sources, lactose has been most widely used in cellulase production. However, lactose induces less cellulase from *T. reesei* than cellulose, and it is not as readily available. Other proposed soluble carbon sources are not economically attractive.

In our studies of cellulase production by *T. reesei*, we have shown that water-soluble acid-hydrolyzed starch is an excellent inducer of these enzymes and induces the enzymes to the same extent as pure cellulose.[15] We have also found that the addition of wheat bran to acid-hydrolyzed starch improves cellulase production. Since pure starch is expensive, the next logical step appeared to be to try treating whole wheat grains and testing the wheat hydrolyzates for their effectiveness as cellulase inducers. This paper reports the results of these trials.

Address reprint requests to Dr. Wayman at the Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A4
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Materials and methods

Substrate preparation

Flours from whole hard wheat and soft wheat were kindly provided by Grain Process Enterprises Ltd. (Scarborough, Ontario, Canada). Natural and raw wheat bran, the product of Sunny Crunch Foods Ltd., Ontario, Canada, was obtained from a local supermarket. Corn starch, α-amylase (EC 3.2.1.1, Termamyl-120L[§]), and glucoamylase (EC 3.2.1.3, AMG-200L[§]), products of Novo Industri, A/S, Copenhagen, Denmark, were the gifts of St. Lawrence Reactors Ltd. (Mississauga, Ontario, Canada). Liquid β-glucosidase (8.95% protein), Novozym 188, was purchased from Novo Industri.

Whole wheat flour slurry at a concentration of 25% dry solids was hydrolyzed with 0.1 to 0.6 M HCl in an autoclave at 121°C for 1 to 2 h. The resulting syrup was chilled and neutralized with calcium hydroxide to pH 5.5. Alternatively, the soft wheat flour slurry at a concentration of 25% dry solids was liquefied with 0.15% α-amylase (on dry solids) in a boiling water bath at pH 6.8 for 20 min under constant stirring. The pH value of the liquefied wheat was then adjusted to 5.5 with 10% HCl.

Acid-hydrolyzed whole hard wheat and soft wheat were designated as AHW1 to AHW6 and ASW1 to ASW6 respectively according to different conditions used for hydrolysis, and enzyme-liquefied soft wheat was designated as ESW. They were used as carbon sources for cellulase production.

Cellulase production

Trichoderma reesei Rut C-30, ATCC 56765, was obtained from the American Type Culture Collection. The procedures used for culture maintenance, inoculum preparation, and cellulase production were as described previously,[7] with the following modifications. The carbon source used for cellulase production was 13.5 g l^{-1} (based on total reducing sugars or total carbohydrate) of specified substrates. The initial pH value for cellulase production was 5.5 and was not adjusted during cellulase production (unless otherwise specified).

Saccharification

The cellulase preparations from 6-day cultures were evaluated by their 48-h saccharification performance on shredded waste office paper (approximately 0.3 × 2.0 cm) in a 9.34% suspension as described previously.[7]

Analytical techniques

Cellulase filter paper activity (IU ml^{-1}) and cellobiase activity (IU ml^{-1}) were determined by the method of the International Union of Pure and Applied Chemistry (IUPAC).[16] Protein in cellulase preparations was precipitated by 10% trichloroacetic acid (TCA) solution and quantified by the colorimetric method of Lowry *et al.*[17] Protein in the wheat samples was analyzed by the standard Kjeldahl method. The calculation of the quantity of protein in the wheat samples was based on total nitrogen content (including nonprotein nitrogen). The starch content in the wheat samples was measured by the enzymatic method of Holm *et al.*[18] Analysis of the office paper and total carbohydrate in the wheat samples was conducted according to the method of Wayman and Parekh.[3] Monosaccharides and disaccharides were analyzed by HPLC (Sugar Analyser I, Waters) with an Aminex HPX-87P column using water at 65°C as an eluent, refractive index detector, and 16 g l^{-1} *n*-propanol as the internal standard. Oligosaccharides were characterized by HPLC on an Aminex-42A column eluted with water at 85°C.

Results and discussion

Substrate analysis

Table 1 shows the composition of raw materials used in the present study. The main component of the wheat samples was starch, which constituted more than 71% of their dry substance. Wheat bran contained nonstarch carbohydrate, which was composed of cellulose, pentosans, and low-molecular-weight sugars. All the raw materials contained high levels of crude protein (12–18%). They also contained fat and ash, which were most of the remainders.

In our previous study, it was found that starch itself was a poor inducer for cellulase production by *T. reesei*, but it was rendered highly effective by acid hydrolysis.[15] This was because acid hydrolysis of starch resulted in the formation of reversion products of glucose, such as sophorose, which have been considered as the "real" inducers of cellulase biosynthesis in *Trichoderma* species.[19, 20] To make the wheat samples water-soluble and inductive, both soft wheat and hard wheat were hydrolyzed using HCl. The hydrolysis was conducted at 121°C in a 25% suspension with varied acid strength and hydrolysis time. The contents of mono- and oligosaccharides in the resulting hydrolyzates are presented in *Table 2*. When the wheat samples were hydrolyzed with 0.1 M HCl, starch was only partially liquefied, and the hydrolyzates (ASW1-2 and AHW1-2) contained low levels of low DP sugars and had high viscosity, indicating that the acid strength of 0.1 M was not high enough to cause extensive hydrolysis of the wheat samples. When the acid strength was raised to 0.3 M, the hydrolysis of starch as well as of the fiber fraction took place more or less completely, resulting in glucose as the principal sugar in these hydrolyzates (ASW3-6 and AHW3-6). When the acid strength was further increased, decomposition of sugars started with prolonged hydrolysis, yielding less total reducing sugar. The composition of the hydrolyzates was less sensitive to the hydrolysis time than to the acid strength. The oligosaccharides in ASW3-6 and AHW3-6 were mainly disaccharides and to a lesser extent trisaccharides. Some of the disaccharides were formed from glucose by reversion during acid hydrolysis. The di- or trisaccharides were not further identified in the present study. Hard wheat contained almost the

Table 1 Composition of raw materials

Sample	Moisture (%)	Starch	Crude protein	Nonstarch carbohydrate	Others
Soft wheat	10.56	71.26	12.42	9.79	6.53
Hard wheat	10.70	71.02	14.76	11.43	2.79
Wheat bran	12.28	19.11	18.24	57.61	5.04

Unit of composition (except moisture) is % of dry substance

Table 2 Content of mono- and oligosaccharides in acid-hydrolyzed whole wheat

Sample	HCl (M)	Hydrolysis time (h)	Glucose	Xylose	Galactose	Arabinose & mannose	Oligosaccharides	Total
Soft wheat								
ASW1	0.1	1	10.40	2.58	–	7.31	64.20	84.49
ASW2	0.1	2	13.43	3.55	–	8.04	95.82	120.84
ASW3	0.3	1	167.90	13.34	3.56	8.98	25.32	219.10
ASW4	0.3	2	166.82	13.40	1.85	8.35	23.94	214.36
ASW5	0.6	1	164.08	12.61	2.89	8.24	22.78	210.60
ASW6	0.6	2	158.63	10.21	3.06	8.33	22.35	202.58
Hard wheat								
AHW1	0.1	1	5.33	–	–	5.79	46.90	57.99
AHW2	0.1	2	10.17	2.31	–	6.57	70.63	89.68
AHW3	0.3	1	140.44	11.78	2.69	8.09	28.24	191.24
AHW4	0.3	2	147.70	11.12	2.55	7.68	22.31	191.36
AHW5	0.6	1	149.36	10.82	2.43	7.37	21.44	191.42
AHW6	0.6	2	142.15	9.37	1.87	6.24	19.96	179.59

Unit of sugars, g l^{-1}

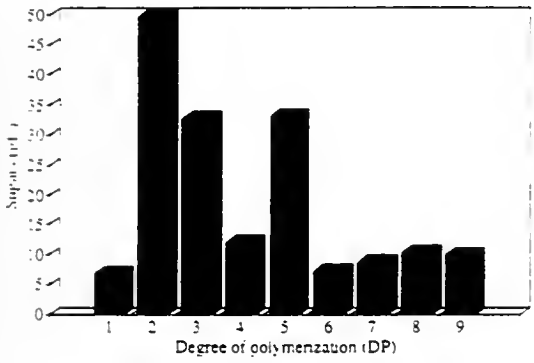

Figure 1 Mono- and oligosaccharides in enzymatically liquefied soft wheat

same level of starch and a higher level of nonstarch carbohydrate compared with soft wheat, and its hydrolyzates contained about 10% lower levels of glucose than those of soft wheat. This could be due to the stronger protein–starch bond in hard wheat, as well as the higher protein level of hard wheat, forming more by-products such as N-substituted glycosylamine between the carbonyl groups of reducing sugars and the amino groups of amino acids or protein during hydrolysis.

Figure 1 illustrates the distribution of oligosaccharides in enzymatically liquefied soft wheat. Maltose, maltotriose, and maltopentose contributed approximately 67% of the oligosaccharides in the liquefied wheat. The degree of liquefaction was more extensive than that prepared by 0.1 M HCl, while the fiber in the wheat remained intact on α-amylase liquefaction.

Cellulase production on acid-hydrolyzed wheat

Different substrates produced from wheat flour by acid hydrolysis under different conditions were evaluated for cellulase production in batch fermentations by *T. reesei* Rut C-30. The evaluation was mainly based on the protein content as well as the saccharification performance of the culture filtrates on waste office paper, which may be a more reliable way than FPU for the evaluation of a cellulase preparation.[7]

Figure 2 illustrates the yields of soluble protein produced by different wheat hydrolyzates. The different preparations of the hydrolyzates dramatically affected the protein yield. For soft wheat, the protein yields increased from about 1.3 to 3.2 g l^{-1} with the increase of acid strength (from 0.1 to 0.6 M) used for the hydrolysis of wheat; for hard wheat, the protein yields increased when the acid strength was raised from 0.1 to 0.3 M and then decreased when the acid strength was further increased. A possible explanation is that the increased acid strength resulted in more reversion products of

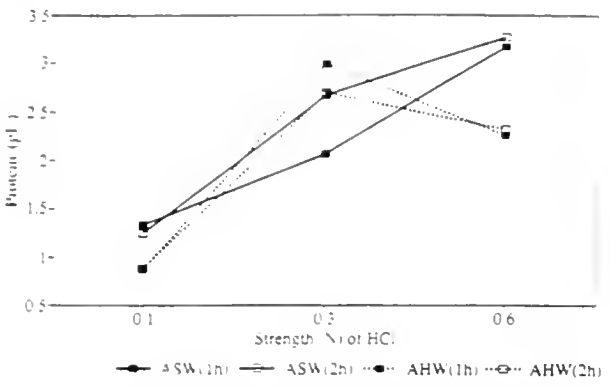

Figure 2 Yields of soluble protein produced by the fungus cultured on whole wheat hydrolyzates prepared using different acid strengths and hydrolysis time. ASW, Acid-hydrolyzed soft wheat; AHW, acid-hydrolyzed hard wheat

glucose in the hydrolyzates which were responsible for the enhanced inductive formation of cellulases. But if the wheat samples were over-hydrolyzed, other by-products were generated which may have served as inhibitors for cellulase production. Hard wheat contained a higher level of protein, possibly forming more such inhibitors upon hydrolysis.

Figure 3a–d demonstrates the efficiency of the culture filtrates in saccharifying shredded waste office paper in a 9.34% suspension. The saccharification efficiency of a cellulase solution was also greatly affected by the nature of the substrate. As indicated by the glucose and cellobiose yields in the saccharification media (*Figure 3a* and *b*), cellulase production on the wheat hydrolyzates prepared using harsh conditions such as 0.6 M HCl, 2 h was preferred. Xylanase activity, as indicated by xylose yields upon saccharification (*Figure 3c*), first substantially increased with increase of acid strength from 0.1 to 0.3 M and then decreased with further increase of the acid strength. Prolonged hydrolysis of the wheat substrates also decreased xylanase synthesis. This indicates that biosynthesis of xylanase was subject to different regulatory routes from those of cellulases, as also reported by other researchers.[21] The overall saccharification ability of the culture filtrates (*Figure 3d*) represented a combination of the activities of different enzyme components in the cellulase solutions. This ability was similarly affected by the nature of the acid hydrolyzate used for enzyme production. Generally, 0.1 M HCl was too dilute for efficient inductive ability; acid strengths of 0.3 to 0.6 M HCl were high enough to generate more effective inducers under conditions used in this research. For soft wheat, the hydrolyzate produced by 2 h hydrolysis using 0.6 M HCl was the best substrate (ASW6) for cellulase production, while for hard wheat, it was more beneficial to conduct the hydrolysis using 0.3 M HCl for 2 h.

The reproducibility of these results was tested by replication of some of these experiments. For example, cellulase production and saccharification performance of cellulases produced using ASW6 was repeated in three runs, with these results: glucose, g l^{-1}, 45.38, 45.53, 42.44; total reducing sugars, g l^{-1}, 66.97, 68.67, 69.10; yield, %, 76.1, 78.03, 78.52. These results are within a percent error of 2 to 4%, which is adequate for comparative purposes.

In the experiments relative to *Figures 2* and *3*, the whole wheat hydrolyzates used for cellulase production still contained a small amount of water-insoluble fiber residue which might have served as an enhancer for cellulase production. Since one of our objectives was to develop a truly soluble substrate, it was necessary to find out whether the soluble fractions of the wheat hydrolyzates were effective in inducing cellulase production. *Figure 4* shows the results of cellulase production on filtered (FAHW4 and FASW6) and unfiltered wheat hydrolyzates (AHW4 and ASW6). As expected, the filtered wheat hydrolyzates were as effective as the unfiltered hydrolyzates in terms of protein content and saccharification performance of the resulting cellulase solution.

Effect of nutrients on cellulase production

An expected advantage of whole wheat flour is that it contains 12–15% crude protein which can serve as nutrients after hydrolysis. Comparative studies of cellulase production on an acid-hydrolyzed hard wheat (AHW4) with omission of peptone in the additional nutrients, with trace elements as the only additional nutrients, and without any additional nutrients were carried out, with the results shown in *Figure 5*. Peptone has been considered as a good nitrogen source and has been used in most research for cellulase production. Peptone (2%) was demonstrated to be able to double β-glucosidase production when cellulase production was based on sulfite pulp.[5] However, the omission of peptone in the wheat-based medium increased protein yield and did not influence the overall saccharification ability of the culture filtrate, although the culture filtrate without peptone resulted in slightly lower glucose and xylose yields upon saccharification than that with peptone. The results suggest that peptone can be omitted if cellulase production is based on wheat-derived substrate. It seemed remarkable that further omission of other nutrients, including KH_2PO_4, urea, $(NH_4)_2SO_4$, $CaCl_2$, and $MgSO_4$, or even including all the trace elements, did not significantly affect the overall saccharification ability of the culture filtrate and increased glucose yield by about 12% in the saccharification medium, while the protein yield and xylanase activity of the culture filtrates were significantly reduced. This may also imply that biosynthesis of different cellulase enzymes takes different regulatory routes and is controlled not only by the nature of a carbon source but also by the presence of nutrients. If one is interested in producing a cellulase mixture with higher cellulase activity and low xylanase activity based on whole wheat-derived substrate, all the additional nutrients can be omitted. The savings are significant because total nutrients usually required for cellulase production amount to 40% of the total carbohydrate weight.

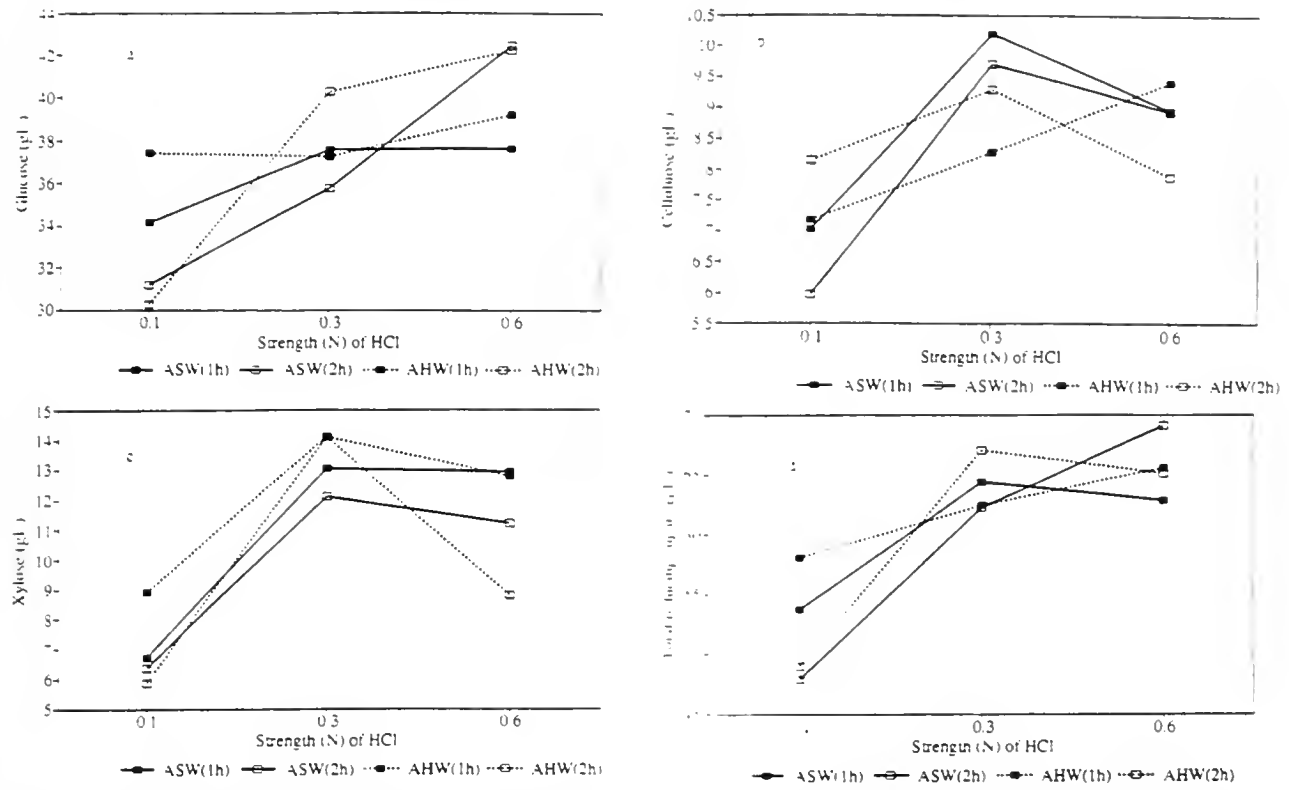

Figure 3 Saccharification performance on shredded office paper of cellulases produced on wheat hydrolyzates using different acid strength and hydrolysis time. a, Glucose yield; b, cellobiose; c, xylose yield; d, yield of total reducing sugars; ASW, acid-hydrolyzed soft wheat; AHW, acid-hydrolyzed hard wheat

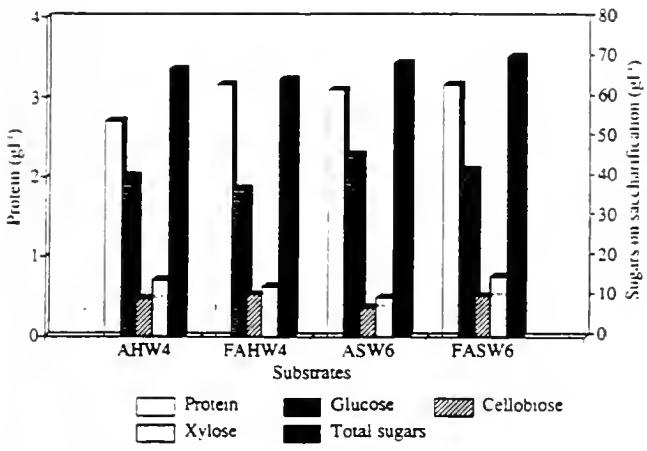

Figure 4 Effect of filtered wheat hydrolyzates on cellulase production and on saccharification performance of the cellulases on shredded office paper. AHW4, Acid-hydrolyzed hard wheat (0.3 M HCl, 2 h); FAHW4, filtered AHW4; ASW6, acid-hydrolyzed soft wheat (0.6 M HCl, 2 h); FASW6, filtered ASW6

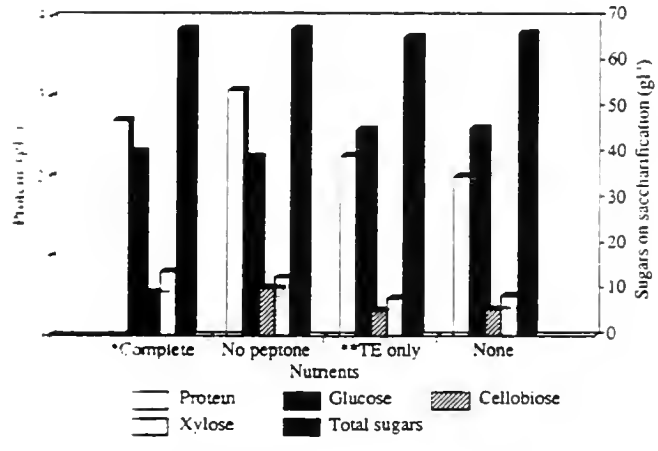

Figure 5 Effect of nutrients on cellulase production and on saccharification performance of the cellulases on shredded office paper. Substrate: acid-hydrolyzed hard wheat (0.3 M HCl, 2 h) AHW4; *complete nutrients: peptone, urea, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, Tween 80 and trace elements; **TE: trace elements including $FeSO_4$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2$, and $MnSO_4 \cdot H_2O$

Effect of pH control on cellulase production

The pH has a significant impact on cellulase production, with an optimum value of between 3.0 and 4.0 in the case of pure cellulose as substrate,[4] and higher in the case of natural cellulosics as substrates.[8] Strict pH control during cellulase production is essential for a successful fermentation, since there is a strong growth-associated drop in pH. The pH value in fermentor cultures can be monitored and regulated by the addition of alkali solution, while the pH in shake flask cultures

may be controlled by the use of a nutrient medium with a high buffering capacity, such as phosphate buffer medium.[22] *Figure 6* shows the effect of pH control with the addition of 0.1 M KH_2PO_4 on cellulase production using whole wheat hydrolyzates (AHW5 and AHW6) in shake flask experiments. As can be seen in *Figure 6*, cellulase production without pH control was marginally better than that with pH control, indicating that the wheat hydrolyzates had high buffering capacity, possibly due to the presence of amino acids and other organic acids. The control of pH during cellulase production was therefore eliminated in this study when whole wheat hydrolyzates were used as substrates. This would facilitate practical operations.

Cellulase production on other wheat substrates

Other wheat substrates, including untreated whole soft wheat flour (SW0) and whole hard wheat flour (HW0), enzymatically liquefied soft wheat flour (ESW), wheat bran, and refined starch were also evaluated for cellulase production in batch cultures of *T. reesei* Rut C-30, with the results shown in *Figure 7*. Comparing these results with those shown in *Figure 4*, it was surprising that untreated wheat flours were as good inducers as their best acid hydrolyzates. Although the culture filtrate resulting from untreated wheat flour contained less protein, it still had high saccharification ability. This means that untreated whole wheat flour produced cellulases of higher specific activity than did acid-hydrolyzed wheat flour. When wheat bran or pure starch was used as the only carbon source, they were much less effective in inducing cellulase production. It seemed that there was some synergistic action of the bran and starch in whole wheat for maximum production of cellulase enzymes. When the starch fraction of the wheat was converted into maltodextrins with α-amylase and then used as substrate (ESW) for cellulase production, it was not very effective in inducing cellulase biosynthesis; however, if a small amount of glucoamylase (0.15% on dry solids) was added to the maltodextrins during cellulase production, cellulase production was greatly improved to a level approaching that obtained on untreated whole hard wheat flour (HW0), suggesting that glucoamylase contributed to the formation of cellulase inducers.

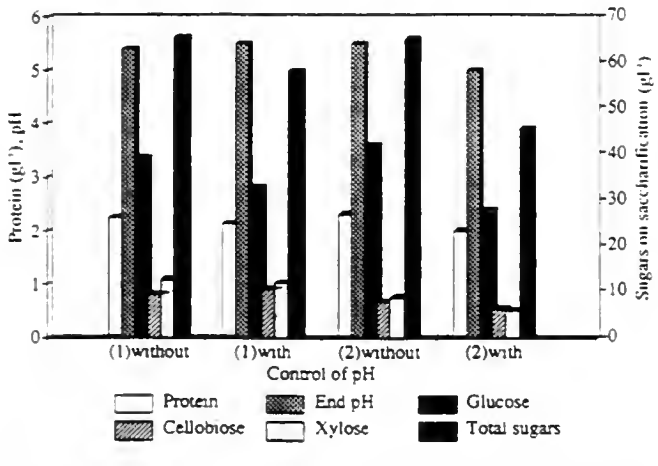

Figure 6 Effect of pH on cellulase production and on saccharification performance of the cellulases on shredded office paper. Substrates: (1) acid-hydrolyzed hard wheat (0.6 M HCl, 1 h) (AHW5) and (2) acid-hydrolyzed hard wheat (0.6 M HCl, 2 h) (AHW6)

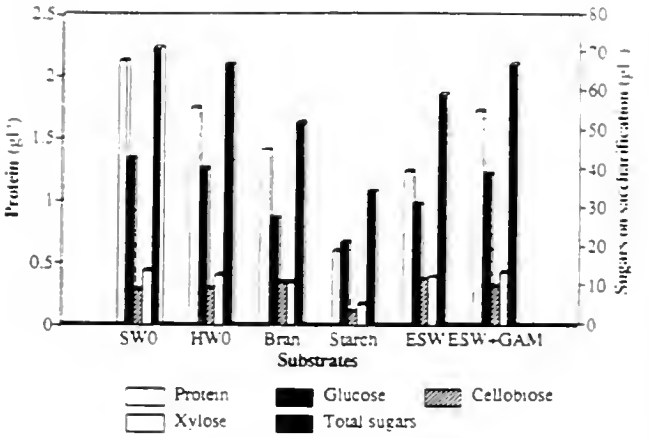

Figure 7 Cellulase production on different wheat substrates and saccharification performance of the cellulases on shredded office paper. SW0, Untreated soft wheat flour; HW0, untreated hard wheat flour; ESW, enzymatically liquefied soft wheat; GAM, glucoamylase

Overall evaluation of cellulase production on wheat hydrolyzates

Cellulase production on whole wheat hydrolyzates prepared with 0.3 to 0.6 M HCl was comparable to that on pure cellulose or the hydrolyzates of pure starch under the same conditions.[15] Cellulases produced from wheat hydrolyzates showed higher specific activity and higher activity forming glucose than those from cellulose according to the saccharification performance of the enzymes.[15] Cellulase activity in terms of an international filter paper unit was 2.52 IU ml^{-1}, and the specific activity was between 0.8 and 0.9 IU mg^{-1} protein when the cells were cultivated on 13.5 g l^{-1} of ASW4 or ASW6; the cellobiase activity was 0.28 IU/ml^{-1} when the enzyme production was carried out using ASW6. When the culture filtrates were directly used for saccharifying 9.34% shredded waste office paper, more than 78% conversion efficiency of the theoretical value was obtained. If a small amount of β-glucosidase (0.01 ml for each gram of dry paper) was supplemented, the culture filtrate could be diluted to 50% of its original strength for the same saccharification efficiency. The saccharification efficiency can be further improved by pretreatment.

Conclusions

Soluble whole wheat hydrolyzates prepared by 0.3 to 0.6 M HCl were effective inducers for cellulase produc-

tion by *T. reesei*. Cellulases produced by *T. reesei* using 1 g whole wheat as carbon source were effective in saccharifying 13.05 g shredded waste office paper, yielding 9.61 g fermentable sugars when supplemented with a small amount of β-glucosidase. The inducing ability of the acid-hydrolyzed whole wheat may be attributed to reversion products of glucose.

Since whole wheat hydrolyzate is water-soluble, the adoption of this carbon source would simplify the operation of cellulase production and allow development of continuous processes using immobilized cells for cellulase production. The wheat hydrolyzate is less expensive than cellulose and is widely available. The high protein content of wheat allows partial or total omission of the usual nutrients required for cellulase production, further lowering the cost of cellulase production. The high buffering capacity of the whole wheat hydrolyzates requires no strict control of pH during fermentation, facilitating practical operations.

Untreated insoluble whole wheat flour was also a good inducer for cellulase production by *T. reesei*. The cellulases produced on whole wheat flour exhibited high specific activity. Comparatively, enzymatically liquefied starch (ESW) was not a good inducer: the addition of glucoamylase to the fermentation medium using ESW as the substrate significantly improved cellulase production.

To our knowledge, this is the first time that cellulases have been produced by *T. reesei* using whole wheat as a carbon source. Further investigations are in progress to obtain a better understanding of the mechanisms controlling biosynthesis and secretion of cellulases on wheat substrates.

Acknowledgement

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